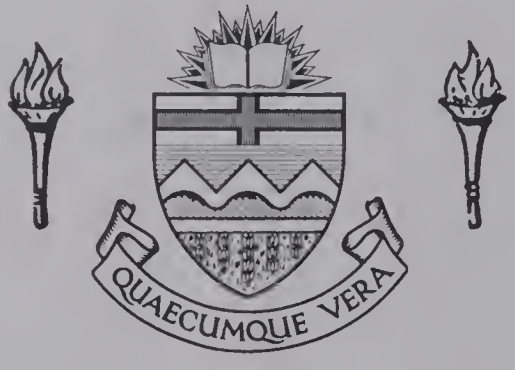

THE UNIVERSITY OF ALBERTA

THE ACTION OF GLYCEROL AND DIMETHYLSULPHOXIDE ON THE FREEZING-THAWING OF RAT UTERINE SMOOTH MUSCLE

by

JIA-HUEY LIN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Action of Glycerol and Dimethylsulphoxide on the Freezing-Thawing of Rat Uterine Smooth Muscle" submitted by Jia-Huey Lin in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Some of the damage which occurs in rat uterine smooth muscle when it is frozen and thawed has been investigated and attempts have been made to improve the methods of protection with the agents glycerol and dimethylsulphoxide by varying the times, temperatures and concentrations of these cryoprotective agents used in the basic procedure (old method).

Horns frozen at -196°C with glycerol (old method) showed normal spontaneous and drug-induced contractions after thawing, thus contraction could not serve to measure the extent of freezing damage. Electrolyte analyses showed the tissue potassium content was a sensitive indicator of damage. Wet weight changes were partly dependent upon the occurrence of osmotic shock during the removal of the glycerol but electrolyte changes were mainly due to freezing damage and not to osmotic shock. Similar results were obtained using dimethylsulphoxide protection.

Dipyridamole at low concentration partially prevented the potassium loss during freezing with glycerol protection and also doubled the ATP content of the thawed uteri.

Measurements of tissue calcium also served to compare the different methods of protecting tissue, and indicated the better survival obtainable with the use of dipyridamole.

Rat uterine horns frozen at -196°C with dimethylsulphoxide (new method) showed very poor contractility after thawing and had a large potassium loss. Dimethylsulphoxide itself in the absence of freezing did not cause significant electrolyte imbalance.

Magnesium sulphate did not protect uteri against freezing-thawing damage and also lacked a synergistic action with glycerol.

Frozen tissue in which solidification occurred was subjected to greater damage than "frozen" tissue in which solidification did not occur.

Uterine wet weight changes in 10% glycerol and 10% dimethylsulphoxide indicated no appreciable difference in the intracellular penetration of these agents. Stepwise changes of concentration also revealed no difference.

ACKNOWLEDGEMENT

To Dr. P. M. Carroll, my supervisor, I extend sincere appreciation for the encouragement, guidance and helpful criticism which she has given me throughout the course of this work.

The drugs: dipyridamole, RE 102 and RA 171, were generously supplied by Dr. Karl Thomae, G.m.b.H, Germany.

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LIST OF ABBREVIATIONS

DMSO:	Dimethylsulphoxide
CPA:	Cryoprotective Agent
PSS:	Physiological Salt Solution
EDTA:	Ethylenediamine tetraacetic acid
ATP:	Adenosine triphosphate

INTRODUCTION

1. Historical Summary

Robert Boyle in the 17th century and Spallanzani in the 18th century were interested in the effects of cold, but there were no really significant advances in knowledge of low temperature biology until the last three decades. Luyet and Gehenio (1940) made extensive observations on the freezing of a wide variety of organisms from bacteria to nematodes and also of tissues isolated from the higher phylogenic orders, notably frog muscles and mammalian erythrocytes. They were convinced that cellular damage and death were caused by ice formation itself and therefore they tried to prevent ice formation by increasing the rate of cooling (Pegg, 1966). In 1949, Polge, Smith and Parkes discovered by accident that 10% glycerol could protect fowl spermatozoa during freezing and thawing (Smith, 1961). This valuable discovery was immediately applied to a variety of cell types and even more important it led to fundamental studies of the mechanisms of freezing damage. Since 1949, cryobiology has been more intensively studied and the knowledge gained is being applied to the long-term storages of tissues and organs.

2. Nature of Damage Caused by Freezing and Thawing

Several mechanisms have been proposed to account for the damage caused by freezing and thawing, but it is probable that no one explanation is the complete answer. The proposed mechanisms include mechanical damage resulting from ice crystal growth; dehydration (also resulting from ice

crystal growth) giving rise to toxic concentrations of electrolytes which may then denature the lipoprotein complex of the membrane; dehydration enabling disulphide bonds to cross link protein groups not normally linked together. These are now discussed in detail.

(i) Mechanical Damage by Ice Crystal Growth

Ice might cause damage either extracellularly or intracellularly.

(a) Extracellular ice formation:

When amoebae were cooled and studied microscopically, it was found that although ice completely surrounded the amoebae, they yet remained viable, providing that intracellular ice formation did not occur. Amoebae can in fact withstand external hydrostatic pressures several hundred times normal. Other cells however may not be so resistant to external pressure (Smith, 1951, 1961).

(b) Intracellular ice formation:

It has often been stated that the structural disorganization and death of cells after intracellular freezing and thawing are the result of mechanical disruption by the intracellular ice crystals (Meryman, 1960a,b, 1966). In fact, when amoebae were cooled and intracellular ice formed, then on thawing, the amoebae did not survive (Smith, 1961).

Luyet and Gehenio (1940) microscopically studied the effects of cooling on various single cells and they assumed that cellular damage was caused by ice formation itself, therefore they suggested that sufficiently rapid cooling by producing vitrification of water instead of crystallization would prevent this damage.

However, proof that vitrification can occur was lacking and is still lacking. In fact, the so called "vitreous" state has been shown by polarization studies actually to be a microcrystalline state (Luyet, 1965).

In addition to damage caused by intracellular ice formation at the time of cooling, it is also possible that the damage occurs during the thawing due to the phenomenon of migratory recrystallization. This consists of the formation of larger crystals, at the expense of smaller ones, by the migration of water molecules and occurs during slow temperature increases taking place below zero (Luyet, 1958, 1965). It is therefore possible that during thawing the small intracellular crystals may transform into larger ones and thus cause damage (Rey, 1960; Mazur, 1960; Luyet, 1965).

(ii) Dehydration Giving Rise to Toxic Concentrations of Electrolytes

As ice forms in a salt solution, the concentrations of electrolytes in the remaining solution increase . Lovelock (1953a,b) gave strong experimental evidence that hemolysis of human erythrocytes by freezing-thawing is due to this high concentration of electrolytes accumulating as the water is removed as ice. Lovelock (1957) also found that a high salt concentration caused a loss of phospholipid from the erythrocyte and on the basis of this evidence suggested that lipid-protein complexes may be the weak link in many of the cells challenged by freezing. He considered these complexes as "inherently unstable and probably maintained in living cells by continuous synthesis."

(iii) Dehydration Enabling Disulphide Bonds to Form

Levitt (1962) has proposed a theory of freezing injury for plant tissue which invokes dehydration as the primary process leading to injury. He proposed that as ice formation proceeded (either intracellularly or extracellularly), the intracellular proteins were brought into close contact (due either to the intracellular ice compressing them or due to decreased cellular volume as water passed out of the cell), and while in this state of close contact -S-S- bonds could form between adjacent -SH groups. On thawing, the tendency would be for the proteins to return to their normal positions, but the newly formed -S-S- bonds would impose changes on the protein structure, probably causing them to unfold. Evidence to support this theory (at least in plants) was obtained by -S-S- and -SH analysis (Levitt, 1962).

3. Cryoprotective Compounds

The cryoprotective compounds can be classified as follows:

(i) Compounds which freely and rapidly penetrate into cells; glycerol and dimethylsulphoxide (DMSO).

Glycerol protects many simple cells and tissues to varying degrees, e.g., spermatozoa, red blood cells, uterus, heart and kidney (Smith, 1961). DMSO protects spermatozoa, erythrocytes, tissue culture cells of several types, mammalian bone marrow and mammalian smooth muscle (Doebellar, 1966; Farrant, 1965b; Lovelock, 1959; Smith, 1961).

(ii) Compounds which depend on a transport system in order to penetrate

the cell; e.g., glucose protects HeLa cells, Escherichia coli and human erythrocytes (Doebellar, 1966).

(iii) Relatively low molecular weight compounds which do not penetrate cells readily; e.g., sucrose, lactose and raffinose which protect HeLa cells, Escherichia coli and human erythrocytes (Doebellar, 1966).

(iv) Relatively high molecular weight compounds too large to penetrate the cells; e.g., dextran, polyvinylpyrrolidone and hydroxyethyl starch. Both dextran and polyvinylpyrrolidone effectively protect erythrocytes under relatively rapid cooling conditions, but protect less effectively or not at all, under slow cooling conditions. Dextran protects at least some microorganisms but only weakly protects tissue culture cells (Doebellar, 1966). Hydroxyethyl starch effectively protects erythrocytes (Knorpp, 1967).

Glycerol and DMSO have been more extensively studied than any of the other cryoprotective agents.

4. Possible Mechanisms for the Protective Action of Glycerol and Dimethylsulphoxide

The action of the cryoprotective agents during freezing-thawing has been explained as preventing or reversing the three afore-mentioned types of damage: thus

(i) prevention of ice crystal formation and growth;

(ii) reduction of concentration of electrolyte;

(iii) prevention of disulfide bond formation.

Glycerol and DMSO both form eutectic mixtures with aqueous solutions (freezing points given in Table I); therefore at temperatures below zero and above the eutectic temperatures, they reduce the quantity of ice which separates from the salt solutions and at suitable concentrations will prevent ice formation. Lovelock (1953b) and Lusena (1956) thought that this might be the reason for their protective activity. Lusena (1960) also reported that glycerol and other cryoprotective agents caused a slower rate of ice crystal growth than occurred in their absence. Therefore at any given temperature with glycerol or DMSO, the concentration of salts will be correspondingly less than would have accumulated in the absence of a cryoprotective agent, and Lovelock (1953a,b) and Farrant (1965b) thought that this partial prevention of salt accumulation might also be the reason for the protective activity.

Levitt (1962) thought that in animal cells the glycerol penetrated intracellularly, where it not only replaced the water molecules and thus prevented the proteins from being compressed together by ice, but also formed hydrogen bonds with the -SH groups, thus forming a protective coat around them and preventing -S-S- bond formation. In the case of plant cells, this protective effect does not occur, because glycerol is apparently secreted into the vacuoles and therefore cannot accumulate intracellularly and protect the protein molecules.

5. Possible Methods of Minimizing Freezing Injury

If the proposed mechanisms of freezing-thawing injury are correct, then attempts to prevent injury to mammalian tissues must aim at preventing

TABLE I. Freezing Points of Glycerol-Water* and Dimethylsulphoxide-Water Solutions†

Glycerol-Water (wt %)	Freezing Point (°C)	Mole Fraction of DMSO	Freezing Point (°C)
0.0	0	0.00	- 0.5
5.0	- 0.6	0.05	- 8
10.0	- 1.6	0.10	-17
15.0	- 3.1	0.15	-45
20.0	- 4.8	0.20	-110
25.0	- 7.0	0.30	-140
30.0	- 9.5	0.40	-125
35.0	-12.2	0.50	-45
40.0	-15.4	0.60	-12
45.0	-18.8	0.70	- 3
50.0	-23.0	0.80	+ 7
55.0	-28.2	0.90	+12
60.0	-34.7	1.0	+17
65.0	-43.0		
66.7	-46.5		
67.1	-45.5		
90.0	- 1.6		
100.0	17.0		

* From Segur (1953)

† From Havemeyer (1966)

the occurrence of dehydration or at preventing the subsequent effects of dehydration when water is removed to form ice. This might be achieved by attention to the following factors; the cooling rate, the thawing rate and the time required for cryoprotective agent penetration.

(i) Cooling Rate: Mazur (1966) found for yeast, without any cryoprotective agent, that there was an optimal cooling rate which gave the greatest cell survival; at higher rates and at lower rates, survival was reduced. Ice first forms outside the cell and water then passes out of the cell due to osmosis, providing the cell wall is sufficiently permeable. If the cooling rate is too fast, then water has not time to pass out and intracellular freezing occurs. But if the cooling rate is too slow, then the cell is dehydrated for too long at any particular temperature and then the damage results from the higher intracellular electrolyte content. Thus the results are very dependent upon the permeability of the cell wall to water.

Mazur (1966) found similar effects with red blood cells and explained: "injury at cooling rates below the optimum is due to exposure to concentrated electrolytes; injury at cooling rates above the optimum is due to intracellular freezing." The optimal rate for erythrocytes differs from the optimal rate for yeast, because of the different permeabilities.

(ii) Thawing Rate: Rapid thawing is essential to prevent ice crystal growth, but it is difficult to achieve if the tissue is thick.

Microwave heating (or diathermy) has been used by Smith with some success in a small number of experiments (Smith, 1961).

(iii) The Time for Intracellular Penetration of Cryoprotective Agent: DMSO has been assumed to penetrate cells more rapidly than glycerol, because of the better survival of certain cells (bovine erythrocytes and chick embryo cells in tissue culture) after freezing-thawing in the presence of DMSO than in the presence of glycerol (Dougherty, 1962; Lovelock, 1959; Porterfield, 1962).

Quantitative data on the penetration of glycerol and of DMSO are very sparse and are collated in Table II. The time taken by cryoprotective agents to diffuse intracellularly depends upon the permeability of the cells and the thickness of the tissues. Farrant (1965a) found that in guinea-pig uteri weighing more than 130 mg, DMSO penetration was not complete after 20 minutes at 37°C, hence he used Taenia coli weighing less than 60 mg to obtain the data in the above table. For the kidney Henderson et al. (1967) used a perfusion rate of 300 ml/hr; at a rate of 100 ml/hr an additional 10 minutes was required to attain equilibrium.

Although the protective action of a cryoprotective agent has generally been considered to be due to its intracellular action, recently Farrant (1967) has stated that it might not be necessary for the agent to penetrate intracellularly but he did not really give any evidence to support this supposition.

TABLE II. Intracellular Penetration of DMSO and Glycerol

Author	Tissue	Temperature (°C)	CPA 14 mM	Permeability Constant (x 10^{-5} cm/sec) K_{in}
Farrant* (1965a)	Guinea-pig Taenia coli	37	DMSO	1.53
		37	Glycerol	0.75
	Guinea-pig Taenia coli	5	DMSO	0.89
			CPA 10% (v/v)	Time to attain steady state (minutes)
Bickis, Kazaks, Finn & Henderson† (1967)	Novikoff hepatoma	37	DMSO	< 1.5
		37	Glycerol	15-20
		22	DMSO	3-5
		22	Glycerol	50
		2	DMSO	10
		2	Glycerol	Did not penetrate
Henderson, Bickis & Edwards (1967)‡	Dog kidney	RT	DMSO	15-20
		0	DMSO	30

* Calculated from intracellular concentration using isotopes.

† Volume change method.

‡ Steady state determination by isotopes.

A method of improving the survival of smooth muscle during freezing-thawing was empirically discovered by Smith (1961) who found that better contractility of uterine horns was obtained by omitting calcium from the solutions, both for smooth muscle frozen to -79°C and for smooth muscle cooled to +5°C. Farrant (1964a,b) in early experiments with guinea-pig uteri and DMSO protection, found they underwent a prolonged contracture on thawing, which could be reversed by adding EDTA or could be prevented by omitting calcium. Similar results were also obtained when the tissues were not frozen (Farrant, 1964a,b). It was thus concluded that the intracellular calcium probably increased during freezing-thawing and during cooling with cryoprotective agent.

A possible method of minimizing freezing injury can be deduced from the following facts. A relationship between erythrocyte survival and the ATP content after erythrocytes had been stored at -20°C with 3.0 M glycerol was reported by Jones, Mollison and Robinson (1957). Dern, Brewer and Wiokowski (1967) found a highly significant correlation between the erythrocyte ATP level and the viability after storage at 4°C. The ATP of blood stored at 4°C normally decreases, yet it could be increased above the initial level by the addition of adenosine and dipyridamole (1×10^{-4} M) which is a synthetic adenosine deaminase inhibitor (Gibson and Lionetti, 1966). Hence one might reasonably deduce that the loss of the ATP of red blood cells during the freezing-thawing process might be prevented by the use of dipyridamole. It has been shown that erythrocyte potassium uptake requires

ATP and that a decrease in ATP level was accompanied by a loss of potassium (Whittam, 1958). Therefore one may deduce that any beneficial effect of dipyridamole on the ATP level of frozen-thawed tissue could probably be detected by an increased intracellular potassium. Experiments to test this deduction will be described.

6. Preservation of Mammalian Cells, Tissues and Organs

Despite not knowing exactly how the cryoprotective agents act nor what the nature of freezing injury is, the simple cells (spermatozoa and red blood corpuscles) have been successfully stored at low temperatures.

Bull spermatozoa were the first to be successfully preserved at -79°C and -196°C with a high concentration of glycerol (Polge, 1957; Smith, 1961) and since then various mammalian spermatozoa (rat, rabbit, ram, stallion, boar, guinea-pig and human) have been successfully preserved (Smith, 1961). The storage temperature used by different workers has varied between -79°C and -196°C, and the concentration of glycerol has also varied with the type of spermatozoa (Sherman, 1965); therefore it is not possible to define one set of optimal conditions of temperature, time and concentration which is applicable to all types of spermatozoa.

Human red blood cells were first protected by glycerol during relatively slow freezing to -79°C and thawing (Smith, 1950) and erythrocytes of various mammalian species have since been successfully preserved (Smith, 1961; Sloviter, 1951a,b, 1952), but glycerol did not protect bovine red cells (Lovelock, 1959). Subsequently DMSO was used by Lovelock (1959) to protect bovine and human red cells and Huggins (1965a) preserved human erythrocytes with 8.6 M DMSO or glycerol using slow freezing and storage

at -85°C for one week, with less than 5% hemolysis after thawing. Erythrocytes could also be preserved by rapid freezing in the presence of glucose (Meryman, 1955). Other blood cells (leukocyte, lymphocyte, platelet, etc.) have also been successfully preserved (Ashwood-Smith, 1965; Cohen and Gardner, 1966). However, again as with spermatozoa, there is no one set of optimal conditions for preservation of erythrocytes of all species.

The plethora of papers on the freezing-thawing of spermatozoa (Smith, 1961; Sherman, 1965) and erythrocytes (Huggins, 1965a,b; Smith, 1961; Tullis, 1958; Tullis and Lionetti, 1966) is in part attributable to the use of artificial insemination and blood transfusions which have created demands for banks of these materials.

More complex tissues and organs have also been frozen and thawed but only with partial success, probably because the optimal conditions of cooling, thawing and cryoprotective agent concentration have not yet been determined. The work on the uterus, kidney and heart will be reviewed.

The uterus: Smith (1961) attempted to freeze-thaw guinea-pig uterus with and without glycerol protection but was not completely successful. Smith (1961) found that after freezing uterine horns either rapidly or slowly in physiological salt solution without glycerol to any temperature between -5°C and -196°C the horns showed neither spontaneous contraction nor response to histamine after thawing. Uterine horns frozen rapidly to -79°C or to -196°C in physiological salt solution containing 20% glycerol showed no signs of viability after thawing, whereas horns which were cooled slowly to -79°C with 20% glycerol protection showed spontaneous rhythmical

contractions but smaller than occurred before freezing, and also responded to histamine. DMSO was used as a cryoprotective agent for guinea-pig uterus by Farrant (1964a,b, 1965b, 1967). The important development by Farrant (1965b, 1967) was his attempt to prevent the electrolyte concentration from increasing as the tissue was cooled. He maintained constant electrolyte concentration during cooling by the gradual increase of DMSO by one of two methods. In his first method, as the temperature was gradually lowered and as ice gradually formed, he added the equivalent volume of appropriate strength DMSO-water so that the volume remained constant and thus the ionic concentrations were unchanged. In his second method, as the temperature was gradually lowered, he increased the concentration of DMSO to prevent the formation of ice. The second method requires sufficient time for the DMSO to penetrate intracellularly, and thus prevent intracellular ice formation (Farrant, 1965b). Farrant assessed the effectiveness of the preservation by the recovery of spontaneous contraction and by the isometric contractile response to histamine (as did Smith, 1961), and found that uterine smooth muscles were only partially protected by DMSO during slow freezing to and thawing from -79°C (Farrant, 1964b). Farrant (1967) has recently published the details of method I; he maintained the volume constant during the cooling by six stepwise additions of DMSO (Table III) and observed the subsequent ultrastructural changes by electron microscopy. This method prevented many of the changes which otherwise would have occurred but the mitochondria showed some remarkable alterations compared with mitochondria of fresh tissue. Because the eutectic temperature of DMSO water is -136°C, Farrant's tissues were not frozen solid at -79°C. If glycerol is used as the protective agent, at -79°C the

tissues do solidify because the glycerol-water eutectic temperature is -46.5°C.

TABLE III. Cooling Procedure of Farrant

Temperature Attained (°C)	DMSO Additions	
	Concentration (M)	Volume (ml)
+37	1.4	1.0
- 5.4	2.1	0.66
- 7.8	2.8	0.50
-15.5	4.2	0.66
-28.9	5.6	0.50
-73.0	7.0	0.40

The Kidney: The successful transplantation of kidneys may account for the present interest of workers in trying to find the conditions for successful freezing and thawing. Initially, glycerol was used as the protective agent (Rivers, 1961), but DMSO has been found to be superior (Barner, 1963, 1965). On reimplantation of kidneys which had been frozen (but probably not solidified) to -50°C or -10°C, with DMSO or glycerol protection and which still contained the protective agent, rapid swelling of the kidneys occurred which was in part due to the osmotic activity of the agent. Intravascular administration of mannitol at the time of reimplantation has been shown to counteract this osmotic effect (Barner, 1965). Despite failure

to preserve and transplant the entire organ, it has been shown that all the different types of cell found in the kidney will survive the freezing-thawing insult (Barner, 1963, 1965; Clady, Barner, Rivers, Haynes, Watkins, 1967; Halasz, Rosenfield, Orloff, Seifert, 1967).

Clady, Barner, Rivers, Haynes and Watkins (1966) investigated the wet weight changes of kidneys perfused between 10°C and 15°C with 15% glycerol in physiological salt solution and found that considerable fluid accumulated (approx. 30% increase in wet weight). This could be almost completely prevented by the inclusion in the perfusate of 5% human serum albumin. Pegg and Farrant (1967) found that the concentration of colloid and the flow rate affected the wet weight of perfused rabbit kidney.

The Heart: Attempts to protect the heart against freezing-thawing injury have been considerably less successful than with uterus, and the workers have concluded (i) glycerol is more toxic than DMSO (Karow and Webb, 1965), (ii) concentrations of DMSO greater than 20% are toxic and do not allow the heart to recover spontaneous contractions (Karow, Carrier and Holland, 1967). This restriction on the concentration of DMSO has restricted the temperature to which the heart can be cooled without solidification occurring. A claim to have successfully cooled a rat heart to -20°C, was subsequently found invalid; the perfusate before entering the heart was at -20°C but the heart itself was 10°C higher (Karow et al., 1967). Reports on heart experiments have lacked many important details, thus it is difficult to accurately assess the state of research.

7. The Aim of This Thesis

The preceding discussion has shown that the reason for and the nature of the damage during freezing-thawing is not fully elucidated, nor are the modes of action of glycerol and DMSO fully understood. In this thesis, some of the damage which occurs in uterine smooth muscle has been investigated, and attempts have been made to improve the method of protection.

The chief findings of this work were (i) Potassium loss was a more sensitive indicator of tissue damage after freezing-thawing than was muscle contractility. (ii) After freezing-thawing with glycerol, potassium and ATP decreased; sodium, chloride and calcium increased. (iii) Wet weight changes associated with use of cryoprotective agents could be reduced by the reduction of osmotic shock. (iv) The potassium and ATP loss due to freezing-thawing could be reduced by the action of an adenosine deaminase inhibitor. (v) A long incubation in DMSO gave better protection than a short incubation. (vi) Frozen tissue in which solidification occurred was subjected to greater damage than "frozen" tissue in which solidification did not occur. (vii) Wet weight experiments did not indicate an appreciable difference between the intracellular penetration of glycerol and DMSO.

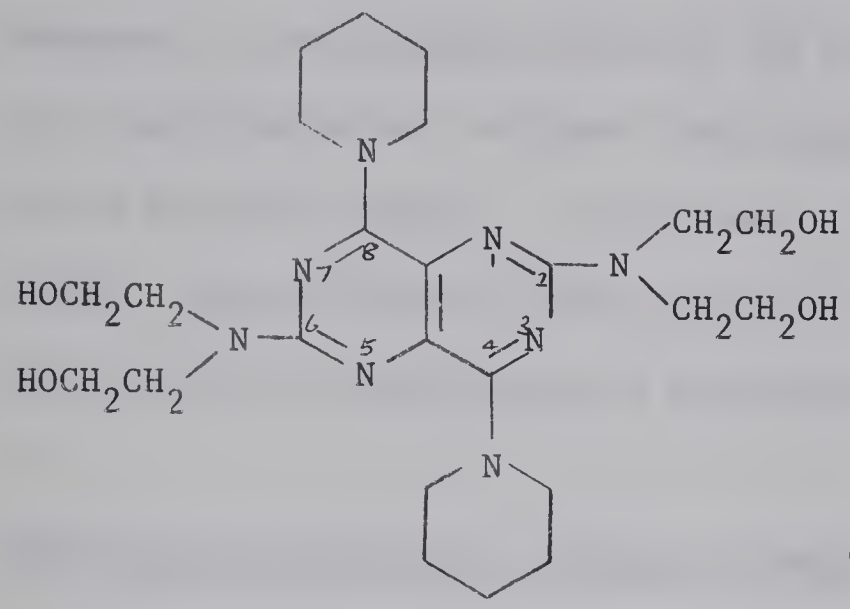
MATERIALS, METHODS AND PROCEDURES

MATERIALS AND METHODS

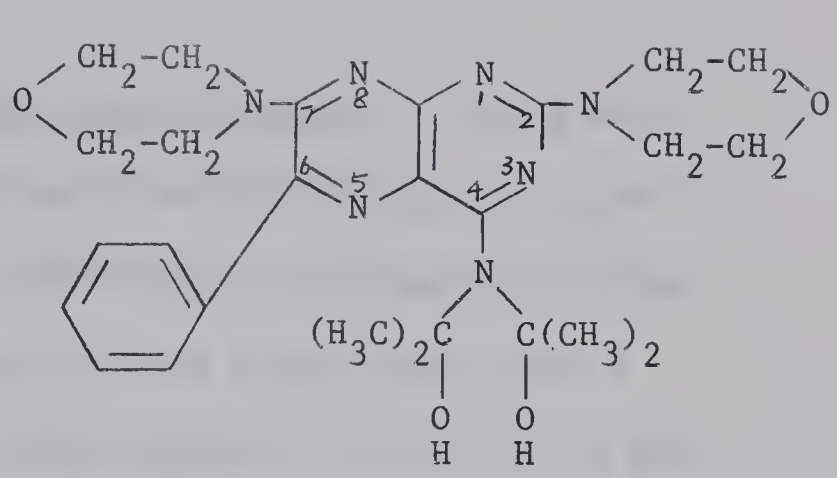
Rats: Female Wistar rats, weight 160-180 g, were treated with 75 µg diethylstilboestrol subcutaneously for each of six days and killed on the seventh day by a blow on the head. Uteri were removed, freed from adhering fat, the horns separated, and cut open lengthwise. The two horns of a single uterus are very similar in behavior, so that the effect of treatment of one horn can be compared with the other horn which serves as control.

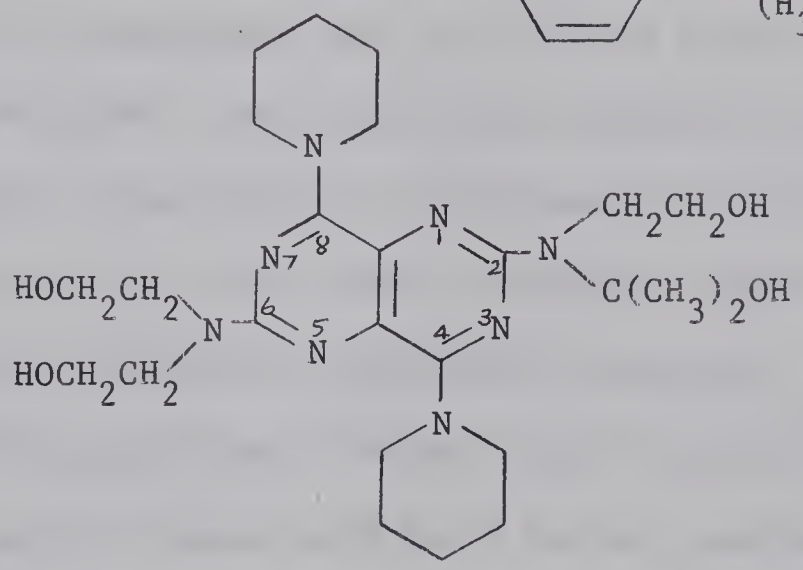
Physiological Salt Solution: The physiological salt solution (PSS) was the same as that used by Farrant (1964b) and contained NaCl (118 mM), KCl (4.5 mM), $CaCl_2$ (1.4 mM), $MgCl_2$ (1.16 mM), NaH_2PO_4 (1.16 mM), $NaHCO_3$ (25 mM) and glucose (11.1 mM). Glycerol or dimethylsulphoxide (DMSO) was incorporated in this solution on a weight per volume basis, except in Procedure 9(iii) which was volume per volume. In all solutions, the ionic concentrations were maintained constant, as given above. All solutions were bubbled with 95% O_2 + 5% CO_2 (giving pH 7.2-7.3) prior to use and those used above 0°C were bubbled whilst in use.

Adenosine Deaminase Inhibitors:



Dipyridamole





RA 171

RE 102

(i) Dipyridamole or persantin; 2,6-bis(diethamolamino)-4,8-dipiperidine-pyrimido-(5,4-d)pyrimidine and its congeners

(ii) RE102; 4-diisopropanolamino-2,7-dimorpholino-6-phenyl-pteridine

(iii) RA171; 2-ethanolisopropanolamino-6-diethanolamino-4,8-dipiperidino-pyrimido-(5,4-d)pyrimidine .

These compounds are only sparingly soluble in water and therefore solutions of each, containing 25 mg/ml were prepared by dissolving the compounds in the minimum volume of 95% ethanol and then gradually diluting with distilled water, and each time incipient turbidity occurred, adding a little more 95% ethanol. In this manner the solutions were made up to volume. These inhibitors were included in PSS-glycerol solutions, such that the final concentration of inhibitor was 10^{-4} M or 5×10^{-6} M.

Electrolyte Estimations by Flame Photometry and Titration: Tissues were dried to constant weight in a desiccator under reduced pressure at room temperature. Portions of dried tissues (30-40 mg) were digested in pyrex digestion tubes with concentrated HNO_3 at 190°C on a sand bath until a white residue was obtained. The residue was dissolved in 10 ml 0.1 N HCl, made up to a suitable volume (usually 50 ml) and the solution analysed for sodium and potassium using an EEL flame photometer, comparing the emission with that of standard solutions of sodium and potassium. Chloride was extracted from 10-20 mg portions of dried tissue by treating with 4 ml 0.1 N HNO_3 containing 10% acetic acid for 2 hrs at room temperature, and then the chloride was estimated using a Buchler-Cotlove chloridometer automatic titrator, comparing the time required for titration with that of standard solutions of chloride. Titrations were performed at medium rate.

Calcium Estimation by Fluorescence Photometry:

Fluorescein Complexone Reagent: A stock solution containing 1 mg/ml of fluorescein complexone (British Drug Houses) in 1.6 N KOH was stored in the dark at 4°C and diluted with 1.6 N KOH to 14 mg/litre, prior to use.

Calcium Standard: 249.7 mg reagent-grade $CaCO_3$ (M.W. 100.91) was dissolved in the minimum amount of 6 N HCl (a few ml) and made up to 100 ml with distilled water, to give 100 mg Ca/100 ml.

2.5 ml of solutions containing calcium or 2.5 ml of water were pipetted into 12 x 75 mm polystyrene tubes, 2.5 ml of fluorescein complexone reagent (14 mg/l in 1.6 N KOH) was added to each, the tubes capped, mixed and allowed to equilibrate at room temperature (23°C) for 10 minutes exactly. The fluorescence was measured in a Turner Fluorometer Model 111 with primary filters no. 110-816 (2A) and no. 110-813 (47B) and secondary filter no. 110-818 (2A-12) such that the exciting light had peak wavelength 436 mμ and the fluorescence longer than 510 mμ was measured by the photomultiplier.

Standard curves were established for calcium in the range 0 to 3 μg/5 ml total final solution. Tissue calcium was estimated using the solutions obtained after nitric acid digestion of the dried tissues.

The accuracy of the method was checked by the addition of known quantities of standard calcium solution to portions of tissue digest solutions and then estimating the amount of calcium added.

ATP Estimations by Luciferin-Luciferase:

Luciferin-Luciferase: The vials of firefly lantern extract, Sigma FLE 50, contain potassium arsenate and magnesium sulphate. In order to obtain a weaker solution of luciferase, but with the normal concentrations of the inhibitor arsenic ion, and the activator magnesium ion, each vial was reconstituted by the addition of 2.5 ml 0.05 M Na_2HAsO_4 (previously adjusted to pH 7.2-7.4 with HCl) and 2.5 ml 0.02 M $MgCl_2$ (previously

adjusted to pH 7.2-7.4 with NaOH) and 5 ml distilled water (Beutler, 1964). Worthington firefly luciferin-luciferase was also reconstituted by the same method.

Standard ATP Solution: A solution of ATP disodium salt-$3H_2O$ (2 mg/ml) was stored frozen and diluted to the required concentration immediately before use. ATP was extracted from the uteri by immersing each horn in 6 ml of boiling water for 10 minutes; the extract was quickly chilled in an ice-bath and made to an appropriate volume (10 ml) with ice-cold distilled water (Strehler, 1957, 1963, 1964). 1.0 ml ATP solution, 1.5 ml water and 0.5 ml buffer were pipetted into a glass tube and the tube placed in a Turner Spectrophotofluorometer adjusted to record all emitted light and with the incident light beam completely masked. 0.5 ml luciferase followed by 0.5 ml air was steadily and rapidly injected with a 1.0 ml syringe below the surface of liquid in the tube. The mode of injection could affect the mixing of the solutions and thus the reproducibility of the light emission (Holmsen, Holmsen and Berhardsen, 1966). Standard curves were established for ATP in the range 0 to 10 µg/3.5 ml total final volumes.

PROCEDURES

1. Tissue Wet and Dry Weights

Immediately after removal from the animal and cleansing, the uterine horns were blotted gently between sheets of filter paper and weighed. At the end of experiments, tissues were blotted and weighed again in order to determine changes of wet weight. The horns were then dried to constant weight at room temperature in a desiccator under reduced pressure to obtain the dry weight.

2. Procedure for Freezing and Thawing Without Cryoprotective Agents

Experimental horns were incubated in PSS at 37°C for 20 minutes, then they were cooled to 0°C and incubated for 30 minutes. The horns were plunged into liquid nitrogen and frozen for 1 hr. Frozen horns were thawed by placing in PSS at 0°C for 30 minutes and then warmed to 37°C and maintained at that temperature for 1.5 hours.

Control horns were similarly treated with respect to time and temperature except that they were not frozen.

3. Old Procedure for Adding Glycerol (Frozen Tissues)

Experimental horns were incubated in PSS containing stepwise increasing concentrations of glycerol at stepwise decreasing temperatures (Table IV), allowing 45 minutes at each temperature, then cooling gradually (approx. 10 min) to the next lower temperature before transferring the tissues to the new concentration of glycerol at that lower temperature.

TABLE IV. Cooling Procedure for Glycerol

(Old Method)

Glycerol/PSS (% w/v)	Incubation Temperature (°C)	Freezing Point of Glycerol/Water (°C)
10	37	- 1.6
20	0	- 4.8
30	- 8	- 9.5

At the end of the final incubation the uterine horns were frozen by plunging them into liquid nitrogen.

4. Old Procedure for Glycerol Removal

(i) With Osmotic Shock

Frozen horns were thawed by immersing in PSS at 37°C and were then transferred to fresh PSS several times during the next twenty minutes, to ensure removal of glycerol, finally allowing 1.5 hours to recover with occasional replacement of PSS.

(ii) Lessening the Osmotic Shock

The frozen horns were immersed in 30% glycerol-PSS at 16°C for 1 or 2 minutes, just sufficient time to thaw them. They were then transferred to 20% glycerol-PSS at 16°C for 10 minutes, then to 10% glycerol-PSS at 16°C for 10 minutes and finally into PSS. The temperature was raised to 37°C during the next 10 minutes and incubation at this temperature continued for an additional 1 hr 20 min. During this time, the PSS was changed every 10 or 15 minutes.

The control horns for procedures 3 and 4 were incubated in PSS without glycerol for the same periods of time and at the same temperatures as for the experimental horns, except that when the experimental horns were incubated below 0°C, the control horns were maintained at zero. This was necessary, because the controls had no glycerol and therefore gross damage would have occurred below zero degrees.

5. Procedure for Adding and Removing Glycerol (Unfrozen Tissues)

Experimental horns were incubated in 10% glycerol-PSS for

0.75 hour at 37°C; cooled to 0°C; incubated in 20% glycerol for 45 minutes and then in 30% glycerol for 45 minutes, all at 0°C. Glycerol was removed by one of the two methods described above.

The control horns were incubated for the same periods of time, and at the same temperatures, but without any glycerol in the PSS.

6. Estimation of Drug-Response of Tissue Frozen-Thawed with Glycerol

After removal of the uterus from the rat, the horns were cut open lengthwise, one horn was used for the control, the other horn was protected with glycerol (Procedure 3) and stored in liquid nitrogen. The frozen horns were thawed and the glycerol removed with osmotic shock (Procedure 4(i)). Both the control and the frozen-thawed horns were subsequently suspended in organ baths (50 ml) in PSS at 37°C and allowed to recover their spontaneous rhythmical contractions. Isotonic contractions were recorded with lightly loaded levers on smoked drums. The organ baths were cooled to 20°C-22°C, so that spontaneous contractions ceased, before testing the responses of the muscles to drugs.

7. Procedure for Modifying the Electrolyte Balance of Frozen-Thawed Tissues

(i) Addition of Adenosine Deaminase Inhibitors

Dipyridamole or RE 102 or RA 171 at a concentration of 10^{-4} M or 5×6^{-6} M was included in each PSS-glycerol solution prior to freezing (Procedure 3), but some tissues were placed in 10% glycerol-PSS containing inhibitors at 0°C and some at 37°C. The horns were stored for 1 hr at -196°C and then thawed (Procedure 4(ii)). For the group treated with 5×10^{-6} M dipyridamole at 37°C initially, the first

thawing solution (Procedure 4(i)) also contained dipyridamole. Control horns were treated in exactly the same way as the experimental horns except that the inhibitors and glycerol were not included in the solutions and the tissues were not frozen in liquid nitrogen.

(ii) Variation of Calcium Concentration

The normal concentration of calcium in the PSS used in this work was 1.4 mM. Experiments were performed using (a) PSS-glycerol solution which was calcium free and (b) PSS-glycerol solution containing 14 mM Ca. These modified calcium solutions were used during the glycerol impregnation (Procedure 3). The solutions used during glycerol removal (Procedure 4(ii)) had normal calcium concentration. The control horns were in solutions containing normal calcium throughout and were not frozen.

To ensure that results were due to alteration of the calcium, additional to the freezing-thawing process, and not simply due to increased calcium concentration, experiments were performed using PSS-glycerol solution containing 14 mM calcium but not freezing-thawing the tissue.

(iii) Addition of Dibucaine

Experimental horns were incubated in 10% glycerol-PSS and then in 20%, 30% glycerol-PSS containing dibucaine prior to freezing (Procedure 3). Two concentrations of dibucaine, 10^{-4} M and 10^{-6} M, were investigated. Control horns received no dibucaine and were not frozen.

8 (i) Procedure for Protecting with Magnesium Sulphate

After removing from the animal, uterine horns were incubated at 37°C in PSS for 10 minutes. The tissues were then incubated at 37°C in PSS containing 1% $MgSO_4 \cdot 7H_2O$ either for 5 minutes, or for 20 minutes. Tissues were then stored in liquid nitrogen for 15 minutes and subsequently thawed by placing in PSS at 37°C. The PSS was replaced by fresh solution several times and incubation continued for a total time of 1.5 hours before testing for contractility.

(ii) Old Procedure for Protecting with Dimethylsulphoxide

Experimental horns were treated as in Procedure (3) for glycerol, but using DMSO concentrations and temperatures as given in Table V for 45 minute periods.

TABLE V. Cooling Procedure for DMSO
(Old Method)

Concentration of DMSO/PSS (% w/v)	Incubation Temperature (°C)	Freezing Point of DMSO/PSS (°C)
10	37	- 3
20	0	- 6.5
30	-10	-14

9 (i) Procedure for Protecting Frozen Tissue with Glycerol (New Method)

This method attempted to lessen the osmotic changes in the uterine horns by smaller stepwise changes of glycerol concentration but the time of incubation in each concentration was shortened from 45 minutes to 15 minutes because of the increased number of steps. The temperatures and concentrations are given in Table VI.

TABLE VI. Cooling Procedure for Glycerol (New Method)

Concentration of Glycerol/PSS (% w/v)	Incubation Temperature (°C)	Freezing Point of Glycerol/Water (°C)
10	0	- 1.6
15	- 3	- 3.1
20	- 5	- 4.8
25	- 7	- 7.0
30	- 9	- 9.5
35	-12	-12.2

The horns were frozen 1 hr at -196°C and then thawed in 35% glycerol/PSS at room temperature and the glycerol removed at this temperature, attempting to lessen the osmotic shock by transferring to a 5% weaker solution of glycerol in PSS every 5 minutes (thus 30, 25, 20, 15, 10, and 5% glycerol-PSS). Finally the horns were transferred to PSS at

37°C for 1.5 hours, changing the PSS four times at 15 min intervals.

Control horns were incubated in PSS without glycerol using the appropriate times and temperatures except that when experimental horns were below 0°C the control horns were at 0°C.

(ii) Procedure for Protecting Frozen Tissue with DMSO (New Method)

The initial incubation in 10% DMSO/PSS was either at 0°C for 5 minutes or at 37°C for 20 minutes followed by cooling to 0°C. The tissues were then incubated in a stepwise increasing concentration of DMSO/PSS at a stepwise decreasing temperature for periods of 5 minutes as in Table VII. Thereafter the procedure was identical with Procedure 9(i).

TABLE VII. Cooling Procedure for DMSO (New Method)

Concentration of DMSO/PSS (% w/v)	Incubation Temperature (°C)	Freezing Point of DMSO/PSS (°C)
10	0 or 37	- 3
15	- 4	- 4
20	- 6	- 6.5
25	- 8	- 8
30	-14	-14
35	-17	-25

(iii) Procedure for Slow Freezing in Dimethylsulphoxide

This procedure simulated Farrant's recent experiment (1967) with respect to temperatures, concentrations and times, but was modified to prevent ice formation instead of compensating for ice formation (Farrant, 1965b). Experimental horns were incubated in 10% DMSO (v/v) at 37°C for 20 minutes, cooled to 0°C within 5 minutes, and subsequently incubated for 5 minute periods at temperatures and concentrations according to Table VIII.

TABLE VIII. Procedure for Slow Cooling

DMSO Concentration (% v/v)	Incubation Temperature (°C)
15	- 5.4
20	- 7.8
30	-14
40	-28
50	-48

After the final incubation, horns in 50% DMSO-PSS were frozen slowly in an insulated container in the cold atmosphere above liquid nitrogen, such that the temperature dropped from -48°C to -70°C in 45 minutes. The frozen mass was stored in liquid nitrogen for 30 minutes and then rapidly thawed by the addition of warm (37°C) 50% DMSO and

then the horns transferred into 40% DMSO/PSS at 0°C for 5 minutes. DMSO was removed by placing the horns for 5 minute intervals in each of the following concentrations of DMSO/PSS; 40, 30, 20, 15, and 10% v/v. Finally the horns were incubated in PSS at 37°C for 1.5 hours.

Control horns were incubated in PSS at 37°C for 20 minutes, then cooled to 0°C, kept at 0°C for 50 minutes, and finally warmed to 37°C and maintained at this temperature for 1.5 hours.

10 (i) Procedure for Adding and Removing Glycerol to Unfrozen Tissue (New Method)

Experimental horns were incubated at 0°C for 15 minute intervals in each of the following concentrations of glycerol; 10, 15, 20, 25, 30, and 35% w/v. The glycerol was then removed by placing the horns for 5 minute intervals at room temperature in each of the following concentrations of glycerol; 30, 25, 20, 15, 10, and 5% w/v. Finally the horns were incubated in PSS at 37°C for 1.5 hours.

Control horns were similarly treated with respect to time and temperature but without glycerol.

(ii) Procedure for Adding and Removing DMSO to Unfrozen Tissue (New Method)

Experimental horns were incubated in 10% w/v DMSO at 37°C for 20 minutes and then cooled to 0°C as quickly as possible. Incubation continued at 0°C for 5 minute intervals in each of the following concentrations; 15, 20, 25, 30, and 35% w/v DMSO. The DMSO was then removed by placing the horns for 5 minute intervals at 0°C in each of

the following concentrations of DMSO; 30, 25, 20, 15, 10, and 5% w/v. Finally the horns were transferred into PSS at 37°C for 1.5 hours.

Control horns were similarly treated with respect to time and temperature but without DMSO.

11. Procedure for Weight Changes of Unfrozen Tissue

(i) In a Constant Concentration of Cryoprotective Agent

The tissues were blotted gently between sheets of Whatman No. 1 filter paper, weighed and placed in an incubation bath and then reweighed at intervals (the weighing process took less than 1 minute). Experimental horns were incubated in 10% glycerol-PSS or 10% DMSO-PSS at room temperature or 0°C for 90 minutes and were then transferred to PSS at 37°C for another 90 minutes, changing the PSS several times.

Control horns were incubated for similar periods of time, at similar temperatures but in PSS without any cryoprotective agent.

(ii) In a Varying Concentration of Cryoprotective Agent

These experiments simulated the concentration changes occurring in freezing-thawing experiments. Tissues were blotted between filter paper, weighed and then incubated in a stepwise increasing concentration of glycerol-PSS or DMSO-PSS at 0°C and then in a stepwise decreasing concentration, at 0°C or room temperature. Finally the horns were transferred to fresh PSS at 37°C (which was renewed several times) for 90 minutes. The tissues were weighed at intervals throughout the experiment.

The control horns were incubated for similar periods of time, at similar temperatures, but in PSS without any cryoprotective agent.

RESULTS

All results in Tables are expressed as mean value ± standard error with the number of experiments in parentheses, except in a few cases where only two experiments were performed; then the mean value ± difference is given.

1. Electrolyte Changes in Tissue Frozen Without Protection

Table IX shows that rapid freezing to -196°C and thawing of unprotected horns resulted in severe ionic changes in the tissue, consisting of a large loss of tissue potassium (88.5%) and a threefold increase in tissue calcium. Such horns cannot contract (Smith, 1961; Farrant, 1964b, 1967).

TABLE IX. Electrolyte and Weight Changes Due to Freezing-Thawing Without Protective Agent

	% Increase of Wet Wt	% Loss of Dry Wt	Electrolytes m moles/kg Dry Wt		
			K	Na	Ca
Control	13.0 ± 7.0 (2)		361 ± 0.2 (2)	607 ± 14.6 (2)	9.0 ± 1.2 (2)
Experi-mental	22.1 ± 2.9 (2)	13.9 ± 3.2 (2)	41 ± 0.0 (2)	1076 ± 57 (2)	26.7 ± 2.0 (2)

2. Contractility and Electrolyte Changes in Tissue Frozen with Glycerol Protection

Frozen-thawed uterine horns protected with glycerol (Procedures 3, 4(i)) showed strong spontaneous rhythmical contractions at 37°C. The subsequent log dose-response curves to acetylcholine and to serotonin at 20-22°C were very similar to those for the central horns (Fig. I; A, C and D). Comparison of the actual size of the contractions of the frozen-thawed horns relative to the control horns, at identical submaximal dose of drug is given in Table X. The contractions obtained after freezing-thawing were independent of the duration (1 hour, 1 day or 21 days) in liquid nitrogen.

Such glycerol protected frozen-thawed horns (Procedures 3 and 4(i)) exhibited a greater increase of wet weight than occurred in the control horns and by comparison with the control horns, there was also some loss of dry weight (Table XI). The freezing-thawing also resulted in an increase of tissue sodium and chloride and a decrease of tissue potassium. It was immaterial whether the horns were stored at -196°C for 1 day, or for 13 days, or for 21 days (Table XI).

Table XII shows that a prolonged recovery period for 3.5 hours instead of 1.5 hours at 37°C after freezing-thawing with glycerol protection did not improve the ionic imbalance. All subsequent experiments therefore had 1.5 hours for recovery.

3. Correlation of Wet Weight and Electrolyte Changes with Osmotic Shock and with Freezing

Table XIII summarizes experimental results correlating wet weight

FIG. 1. The log dose-response curves of control horns (o) and frozen-thawed horns (•) protected with

A. Glycerol (old method), stored for 3 weeks
B. DMSO (old method), stored for 1 hour
C. Glycerol (old method), stored for 1 week
D. Glycerol (old method), stored for 3 weeks

FIGURE 1.

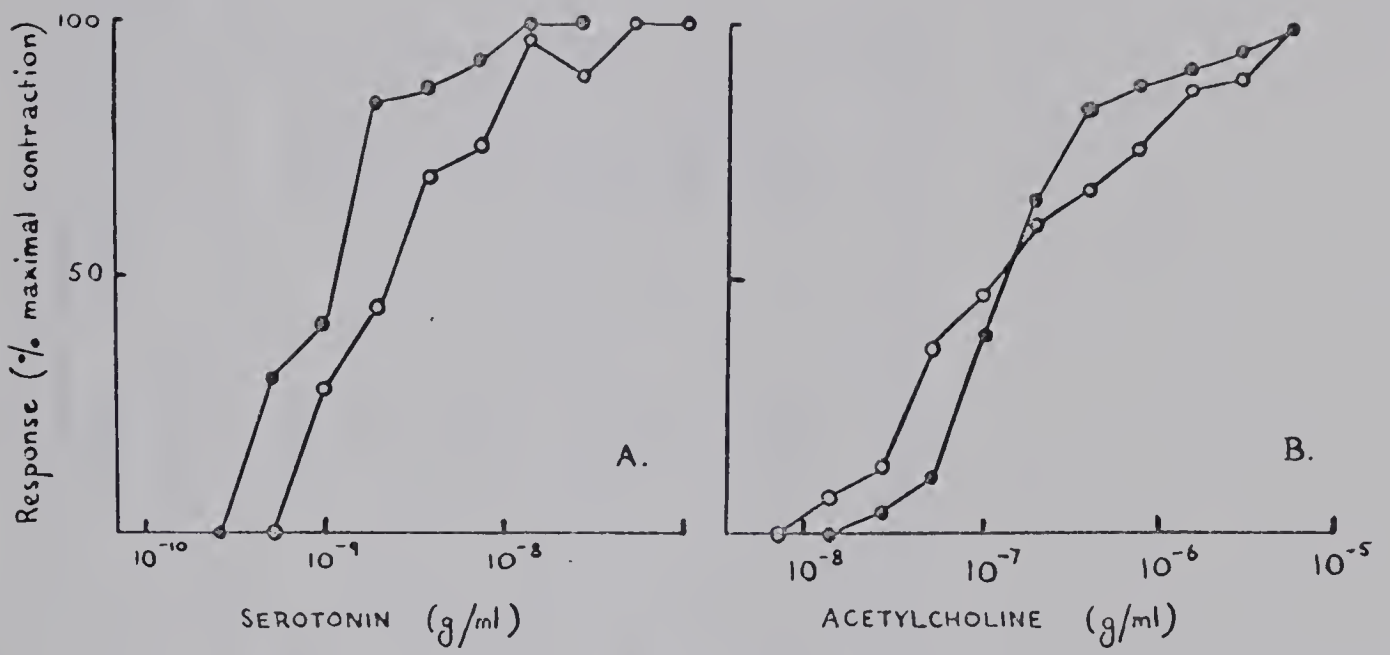

Response (% maximal contraction)
100
50
A.
10^{-10}
10^{-9}
10^{-8}
SEROTONIN (g/ml)
B.
10^{-8}
10^{-7}
10^{-6}
10^{-5}
ACETYLCHOLINE (g/ml)
Response (% maximal contraction)
100
50
C.
10^{-7}
10^{-6}
10^{-5}
ACETYLCHOLINE (g/ml)
D.
10^{-8}
10^{-7}
10^{-6}
10^{-5}
ACETYLCHOLINE (g/ml)

TABLE X. Size of Contraction of Control and Frozen-Thawed Horns

Cryoprotective Agent	Procedure	Drug	Dose (µg/50 ml)	Contraction of Horns (cm/cm)	
				Control	Frozen-Thawed
Glycerol	Old*	Acetylcholine chloride	205	0.10 (2)	0.18 (2)
Glycerol	Old*	Serotonin creatinine sulphate	0.8	0.16 (2)	0.13 (2)
DMSO	Old†	Acetylcholine chloride	320	0.16 (2)	0.10 (2)
DMSO	New‡	Acetylcholine chloride	640	0.17 (2)	0.03 (2)

* Procedures 3 and 4(i), with osmotic shock.

† Procedure 8(ii), reduced osmotic shock.

‡ Procedure 9(ii), reduced osmotic shock.

TABLE XI. Analyses of Glycerol-Protected* Uterine Horns Stored at -196°C for Varying Periods

	Length of Storage at -196°C	% Increase Wet Weight	% Loss Dry Weight	Electrolytes m mole/kg Dry Weight		
				K	Na	Cl
Control-PSS	--	9.7 ± 2.7 (4)		325 ± 9.6 (4)	609 ± 35 (4)	635 ± 35 (4)
Glycerol-PSS	1 day	23.5 ± 2.1 (4)	7.0 ± 3.0 (4)	192 ± 19 (4)	935 ± 52 (4)	768 ± 14 (4)
Control-PSS	--	9.5 ± 4.1 (2)		337 ± 31 (2)	625 ± 12 (2)	579 ± 63 (2)
Glycerol-PSS	13 days	18.2 ± 5.0 (2)	8.3 ± 2.7 (2)	120 ± 7 (2)	1230 (1)	852 ± 82 (2)
Control-PSS	--	8.6 ± 4.6 (2)		312 ± 6 (2)	622 ± 25 (2)	542 ± 25 (2)
Glycerol-PSS	21 days	20.2 ± 0.5 (2)	7.5 ± 2.9 (2)	211 ± 5 (2)	884 ± 58 (2)	725 ± 45 (2)

* Osmotic shock occurred during the removal of glycerol.

TABLE XII(A). The Effect of a Prolonged Recovery Period after Freezing and Thawing with Glycerol

	Length of Recovery Period (hours)	% Increase Wet Weight	% Loss Dry Weight	Electrolytes m mole/kg Dry Weight	
				K	Na
Control-PSS	1.5	9.0 ± 0.8 (8)		358 ± 44 (10)	587 ± 16 (11)
Glycerol-PSS	1.5	17.4 ± 1.9 (8)	8.3 ± 0.9 (11)	165 ± 35 (10)	917 ± 28 (11)
Control-PSS	3.5	15.3 ± 3.8 (4)		308 ± 16 (4)	575 ± 30 (4)
Glycerol-PSS	3.5	24.8 ± 4.8 (4)	8.6 ± 0.8 (4)	148 ± 19 (4)	877 ± 35 (4)

TABLE XII(B).

Recovery Time (hours)	Changes of Electrolyte, as % of Control Value	
	K Loss	Na Gain
1.5	54.5 ± 2.7 (10)	69.4 ± 4.9 (11)
3.5	53.1 ± 6.0 (4)	53.2 ± 3.9 (4)

TABLE XIII. Electrolyte and Weight Changes Correlated with Osmotic Shock and with Freezing

	Incubation Medium	Method of Glycerol Removal	% Increase Wet Weight	% Loss Dry Weight	Electrolytes m mole/kg Dry Weight			
					K	Na	Cl	Ca
Frozen-Thawed Experiments (Procedures 3,4(i & ii))	Control-PSS	--	9.4 ± 1.1 (8)		324 ± 6 (8)	616 ± 22 (8)	598 ± 23 (8)	--
	Glycerol-PSS	Osmotic Shock	21.3 ± 1.4 (8)	8.1 (8)	179 ± 16 (8)	962 ± 42 (8)	778 ± 20 (8)	--
	Control-PSS	--	9.0 ± 0.8 (8)		358 ± 44 (10)	587 ± 16 (11)	558 ± 16 (11)	10.7 ± 0.5 (4)
	Glycerol-PSS	Reduced Osmotic Shock	17.4 ± 1.9 (8)	8.3 (8)	165 ± 35 (10)	919 ± 28 (11)	802 ± 20 (4)	21.1 ± 0.4 (4)
Unfrozen Experiments (Procedure 5)	Control-PSS	--	9.7 ± 1.2 (7)		357 ± 9.5 (7)	528 ± 28 (7)	501 ± 15 (7)	--
	Glycerol-PSS	Osmotic Shock	19.4 ± 2.7 (7)	-3.4 (7)	328 ± 21 (7)	559 ± 36 (7)	583 ± 2 (7)	--
	Control-PSS	--	8.7 ± 3.2 (4)		323 ± 16 (4)	578 ± 14 (4)	534 ± 25 (4)	
	Glycerol-PSS	Reduced Osmotic Shock	12.7 ± 2.0 (4)	1.7 (4)	278 ± 20 (4)	651 ± 8 (4)	564 ± 20 (4)	

and electrolyte changes, with the degree of osmotic shock, and also correlating these changes in frozen and unfrozen tissue. By lessening the osmotic shock during glycerol removal from frozen-thawed horns (Procedure 4(ii)), one could partially prevent the increase of wet weight but could not improve the electrolyte imbalance. Even unfrozen horns showed large wet weight changes when glycerol was removed from them with osmotic shock, but they did not exhibit any appreciable electrolyte imbalance, and their wet weight changes were also partially prevented by lessening the osmotic shock.

4. Modification of Electrolyte Balance by:

(i) Effect of Adenosine Deaminase Inhibitors on Frozen-Thawed Tissues

(a) Electrolyte changes:

In the experiments attempting to improve the electrolyte imbalance, adenosine deaminase inhibitors (dipyridamole, RE 102 and RA 171), at concentrations of 10^{-4} M, did not improve tissue potassium content and RA 171 was definitely detrimental. However, reducing the concentration of dipyridamole to one-twentieth, 5×10^{-6}M, and adding it to tissue initially at 37°C, markedly improved the potassium content, so that the loss was only 32% instead of 68% and this improvement was only in part due to the temperature change since with 10^{-4} M dipyridamole at 37°C, the potassium loss was 55% (Table XIV (A) and (B)).

(b) ATP changes:

In frozen-thawed horns protected by glycerol (Procedure 3 and 4(ii)), the ATP content was 25% of that of control unfrozen horns

TABLE XIV(A). Effects of Dipyridamole and Its Congeners on Electrolyte and Weight Changes of Glycerol-Protected Frozen-Thawed* Horns

	Adenosine Deaminase Inhibitor	% Increase Wet Weight	% Loss Dry Weight	Electrolytes m mole/kg Dry Weight			
				K	Na	Cl	Ca
Control-PSS	--	8.6 ± 1.2 (4)		400 ± 18 (4)	491 ± 58 (4)	577 ± 22 (4)	--
Glycerol-PSS	Dipyridamole 10^{-4}M†	20.3 ± 1.8 (4)	9.2 ± 1.0 (4)	182 ± 14 (4)	724 ± 49 (4)	814 ± 40 (4)	
Control-PSS	--	6.8 ± 1.4 (8)		377 ± 14 (8)	537 ± 39 (8)	472 ± 13 (4)	9.7 ± 0.5 (4)
Glycerol-PSS	Dipyridamole 5×10^{-6}M†	12.5 ± 1.9 (8)	8.7 ± 1.5 (8)	257 ± 15 (8)	843 ± 71 (8)	701 ± 20 (4)	17.2 ± 1.4 (4)
Control-PSS	--	13.2 ± 1.9 (4)		302 ± 5.6 (4)	565 ± 23 (4)	531 ± 16 (4)	--
Glycerol-PSS	Dipyridamole 10^{-4}M	29 ± 5.8 (4)	10.1 ± 1.1 (4)	97 ± 6.9 (4)	995 ± 26 (4)	834 ± 20 (4)	--
Control-PSS	--	10.3 ± 1.5 (4)		314 ± 9.9 (4)	574 ± 15 (4)	568 ± 18 (4)	--
Glycerol-PSS	RE 102 10^{-4}M	17.7 ± 1.2 (4)	5.1 ± 0.9 (4)	106 ± 1.8 (4)	889 ± 6.8 (4)	730 ± 4 (4)	--

(TABLE XIV(A) continues on page 43)

TABLE XIV(A) continued.

	Adenosine Deaminase Inhibitor	% Increase Wet Weight	% Loss Dry Weight	Electrolytes m moles/kg Dry Weight			
				K	Na	Cl	Ca
Control-PSS	--	11.5 ± 2.6 (4)		324 ± 14 (4)	492 ± 26 (4)	496 ± 14 (4)	--
Glycerol-PSS	RA 171 10^{-4}M	20.6 ± 1.7 (4)	9.2 ± 1.4 (4)	68 ± 6.8 (4)	951 ± 17 (4)	753 ± 20 (4)	--

TABLE XIV(B).

Deaminase Inhibitor	Changes of Electrolytes, as % of Control Values		
	K Loss	Na Gain	Cl Gain
Dipyridamole† 10^{-4}M	54.8 ± 1.8 (4)	55.9 ± 8.9 (4)	51.2 ± 11.8 (4)
Dipyridamole† 5×10^{-6}M	31.6 ± 4.0 (8)	50.9 ± 9.2 (8)	48.9 ± 6.2 (8)
Dipyridamole 10^{-4}M	67.7 ± 2.7 (4)	76.4 ± 6.3 (4)	57.3 ± 2.5 (4)
RE 102 10^{-4}M	66.7 ± 0.9 (4)	55.4 ± 4.0 (4)	32.5 ± 4.6 (4)
RA 171 10^{-4}M	78.9 ± 2.4 (4)	86.2 ± 2.4 (4)	47.5 ± 3.6 (4)

* With osmotic removal of glycerol.

† First step performed at 37°C.

(Table XV). However, when dipyridamole at a concentration of 5×10^{-6} M was included in glycerol solution (Procedure 7(i)), the final ATP content was 50% of control value (Table XV). The dipyridamole-treated frozen-thawed horns exhibited spontaneous contractions but measurements were not made.

TABLE XV. Effect of Dipyridamole on ATP Content of Frozen-Thawed Horns*

	ATP µg/g Wet Wt	
	Control†	Frozen-Thawed
Dipyridamole (5×10^{-6} M)	446 ± 33 (4)	204 ± 11 (4)
Without Dipyridamole	424 ± 5.5 (4)	102 ± 14 (4)

* Protected with glycerol

† No dipyridamole was used in the controls, and initial incubation was at 37°C.

(ii) Effect of Calcium Concentration on Frozen-Thawed Tissue

The results of Table XVI show that a high calcium concentration in the glycerol-PSS resulted in increased damage to the tissues after freezing-thawing as measured by ionic changes, but that high calcium itself did not cause ionic imbalance when the horns were not frozen.

TABLE XVI(A). The Effect of Calcium Concentration of the Glycerol-PSS on Electrolyte and Weight Changes of Frozen and Unfrozen Horns

	Calcium Concentration of PSS-Glycerol	% Increase Wet Weight	% Loss Dry Weight	Electrolyte m mole/kg Dry Weight		
				K	Na	Cl
Frozen-Thawed	Control-PSS (1.4 mM Ca)	13.8 ± 1.5 (4)		333 ± 21 (4)	568 ± 21 (4)	547 ± 4.8 (4)
	Ca-Free, PSS-Glycerol	33.0 ± 4.8 (4)	11.9 ± 0.6 (4)	106 ± 6.2 (4)	1147 ± 47 (4)	874 ± 20 (4)
	Control-PSS (1.4 mM Ca)	8.9 ± 1.5 (7)		358 ± 13 (7)	548 ± 14 (7)	558 ± 12 (4)
	Normal Ca, PSS-Glycerol (1.4mM)	18.5 ± 1.8 (7)	9.4 ± 1.6 (7)	166 ± 9.8 (7)	975 ± 37 (7)	802 ± 19 (4)
	Control-PSS (1.4 mM Ca)	11.1 ± 1.3 (8)		344 ± 5.4 (8)	606 ± 18 (8)	580 ± 11 (4)
	High Ca, PSS-Glycerol (14mM)	23.1 ± 1.9 (8)	10.7 ± 2.4 (8)	48 ± 3.3 (8)	971 ± 32 (8)	882 ± 11 (4)
Unfrozen	Control-PSS (1.4 mM Ca)	15.1 ± 1.2 (4)		279 ± 9.8 (4)	671 ± 31 (4)	--
	High Ca, PSS-Glycerol (14mM)	18.2 ± 1.5 (4)	-0.7 (4)	234 ± 11 (4)	719 ± 18 (4)	--

TABLE XVI(B).

Calcium Concentration of PSS-Glycerol	Change in Electrolyte, as % of Control Values		
	K Loss	Na Gain	Cl Gain
Ca-Free	69.8 ± 3.5 (4)	102.7 ± 9.8 (4)	60.0 ± 6.7 (4)
Normal Ca (1.4 mM)	54.5 ± 2.7 (7)	78.0 ± 4.9 (7)	43.7 ± 4.3 (7)
High Ca (14 mM)	86.0 ± 0.9 (8)	62.7 ± 7.8 (8)	52.0 ± 2.9 (8)

The omission of calcium from the glycerol-PSS could not prevent potassium loss and sodium and chloride gain in frozen-thawed tissue, and in fact the omission of calcium caused a worsening of the ionic balance, but this was not nearly so detrimental as the effect of a high calcium concentration. The tissue frozen-thawed without calcium showed a greatly increased wet weight (33%), compared with those in normal calcium (1.4 mM) and associated with this water increase was a greater increase of sodium and chloride.

(iii) Effect of Magnesium Sulphate on Frozen-Thawed Tissue

$MgSO_4$ by itself was completely unsuccessful as a cryoprotective agent for tissue frozen to -196°C because the thawed tissues were unable to contract. When $MgSO_4$ (0.5% ≅ 42 mM) was included in all three glycerol solutions (10, 20, and 30%) prior to freezing, then the ionic balance after thawing was considerably changed, and the low content of potassium (59 m mole/kg dry wt) was very similar to the low potassium (48 m mole/kg dry wt) obtained when using a high concentration of calcium (14 mM) (Tables XVI and XVII).

TABLE XVII. The Effect of Magnesium Sulphate on Electrolyte Balance of Frozen Horns

Old Glycerol Method* + 42 mM $MgSO_4$	% Increase Wet Wt	Electrolyte m mole/kg dry wt	
		K	Na
$MgSO_4$ in 30% glycerol	14.4 ± 0.8 (4)	165 ± 7.9 (4)	792 ± 28 (4)
$MgSO_4$ in 10, 20, 30% glycerol	15.3 ± 2.9 (4)	59.2 ± 4.5 (4)	970 ± 23 (4)

* Procedure 3 and 4(ii)

Thus $MgSO_4$ also lacked a synergistic cryoprotective activity with glycerol.

(iv) Effect of Dibucaine on Frozen-Thawed Tissue

Dibucaine at two concentrations, 10^{-4} M and 10^{-6} M, did not improve the electrolyte imbalance (Table XVIII).

5. Contractility and Electrolyte Changes in Tissue Frozen with DMSO Protection

Uterine horns frozen-thawed with DMSO using the old method (Procedure 8(ii)) were able to contract both spontaneously (Fig. 2,A) and in response to acetylcholine. The log dose-response curves of control and frozen horns were similar (Fig. 1,B) and the size of contractions was also similar (Table X), but these DMSO protected horns were found to have electrolyte imbalance and the calcium showed a twofold increase (Table XIX). Thus there was very little difference between the cryoprotective ability of glycerol and of DMSO as estimated by the electrolyte changes and contractility when using the old methods (Tables XII, XIX and X).

6. The New Methods

(i) Wet Weight and Electrolyte Changes

The new method for glycerol, using smaller concentration changes to lessen osmotic shock (Procedure 9(i)), gave no improvement in the electrolyte balance after freezing-thawing the tissues (Table XX) and in fact the tissue potassium was a little lower than with the old method.

TABLE XVIII. The Effect of Dibucaine on Glycerol-Protected Frozen-Thawed Horns

	Concentration of Dibucaine	% Increase Wet Weight	% Loss Dry Weight	Electrolyte m mole/kg Dry Weight	
				K	Na
Control-PSS	--	18.8 ± 1.0 (4)		313 ± 14 (4)	554 ± 16 (4)
Glycerol-PSS	10^{-6} M	20.1 ± 1.9 (4)	11.6 ± 0.3 (4)	144 ± 3.5 (4)	802 ± 24 (4)
Control-PSS	--	13.2 ± 1.0 (4)		336 ± 5.6 (4)	523 ± 32 (4)
Glycerol-PSS	10^{-4} M	22.4 ± 0.3 (4)	8.7 ± 1.0 (4)	105 ± 21 (4)	875 ± 15 (4)

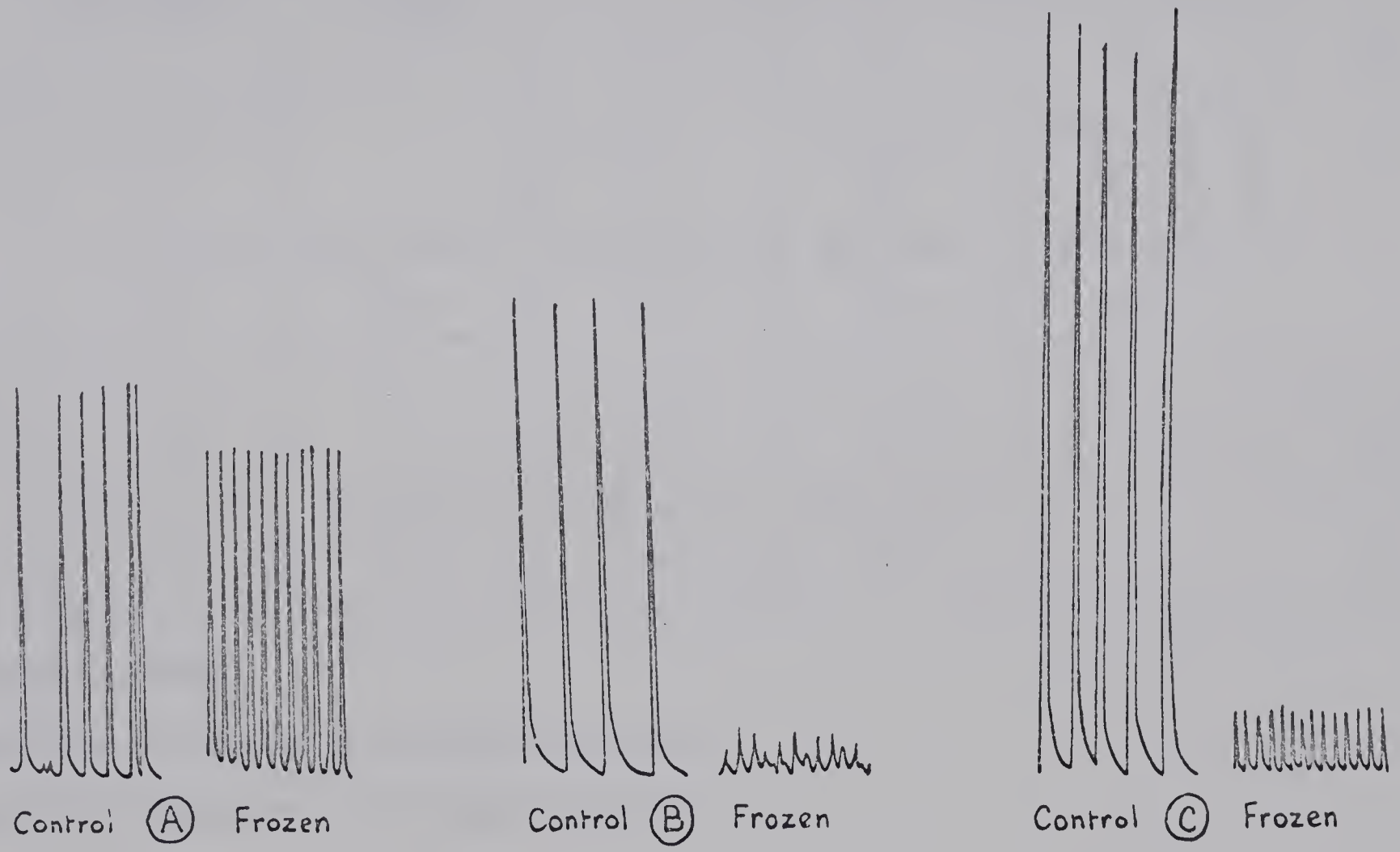


FIG. 2. Comparison of spontaneous contractions of control horns and horns frozen-thawed by (A) old DMSO method; (B) & (C) new DMSO method.

TABLE XIX. Electrolyte and Weight Changes of DMSO-Protected Frozen-Thawed* Horns
(Old Method, Procedure 8(ii))

	% Increase Wet Weight	% Loss Dry Weight	Electrolyte m mole/kg Dry Weight			
			K	Na	Cl	Ca
Control-PSS	13.3 ± 0.9 (3)		350 ± 12 (3)	564 ± 12 (3)	595 ± 49 (3)	--
DMSO-PSS†	19.3 ± 1.2 (3)	8.8 ± 0.4 (3)	143 ± 13 (3)	893 ± 40 (3)	811 ± 17 (3)	--
Control-PSS	5.8 ± 2.4 (4)		354 ± 16 (4)	545 ± 13 (4)	--	10.3 ± 0.2 (4)
DMSO-PSS‡	10.4 ± 3.9 (4)	8.2 ± 0.9 (4)	168 ± 5.7 (4)	745 ± 41 (4)	--	21.2 ± 1.0 (4)

* Osmotic removal of DMSO.

† DMSO concentration increased at 30 minute intervals.

‡ DMSO concentration increased at 45 minute intervals.

TABLE XX. Electrolyte and Weight Changes Due to the New Glycerol Method

	Incubation Medium	% Increase Wet Weight	% Loss Dry Weight	Electrolyte m mole/kg Dry Weight		
				K	Na	Ca
Frozen-Thawed Experiments	Control-PSS	12.6 ± 0.8 (6)		316 ± 8.0 (6)	507 ± 33 (6)	9.0 ± 0.0 (6)
	Glycerol-PSS	25.2 ± 1.7 (6)	6.8 ± 0.9 (6)	111 ± 1.3 (6)	838 ± 12 (6)	28.0 ± 0.9 (6)
Unfrozen Experiments	Control-PSS	3.6 ± 1.9 (4)		373 ± 23 (4)	504 ± 26 (4)	10.1 ± 0.4 (4)
	Glycerol-PSS	10.3 ± 2.3 (4)	4.5 ± 0.9 (4)	304 ± 24 (4)	625 ± 31 (4)	16.7 ± 1.8 (4)

The new method for DMSO (Procedure 9(ii)) resulted in a loss of potassium (Table XXI) which was considerably greater than with the old method (Table XIX). This bad result was not due to the DMSO itself, because when used with unfrozen tissue it did not significantly change the electrolytes (Table XXI).

The final modification to the method (Procedure 9(iii)) based on Farrant's most recent experiment and involving slow cooling to -70°C, was not a success in terms of electrolyte balance (Table XXI).

(ii) Contractility Changes

The contractility of horns protected by the new procedure with DMSO was found to be impaired (Fig. 2,B,C). Although the frequency of the spontaneous contraction was somewhat faster than that of the control horns, both spontaneous contractions (Fig. 2,B,C) and the contractions in response to acetylcholine were very much smaller than occurred in the control horns and very much smaller than occurred in horns protected by the old procedure with DMSO (Fig. 2,A).

7. Wet Weight Changes

(i) A Constant Concentration of Cryoprotective Agent

Fig. 3 and Fig. 4 compare the action of 10% glycerol and 10% DMSO on the wet weight of tissues incubated at room temperature (24°C) and at 0°C for 90 minutes. Control horns, incubated without cryoprotective agent for 90 minutes, showed a 4% increase of wet weight at room temperature and a 16% increase at 0°C, which could be reversed

TABLE XXI. Electrolyte and Weight Changes Due to the New DMSO Method

	Incubation Medium	% Increase Wet Weight	% Loss Dry Weight	Electrolyte m mole/kg Dry Weight		
				K	Na	Ca
Frozen-Thawed Experiments	Control-PSS	8.7 ± 1.7 (2)		308 ± 7 (2)	561 ± 32 (2)	11.8 ± 0.2 (2)
	DMSO-PSS*	11.4 ± 2.6 (2)	6.7 ± 2.3 (2)	73 ± 5 (2)	855 ± 9 (2)	22.1 ± 0.1 (2)
	Control-PSS	9.9 ± 1.9 (4)		383 ± 22 (4)	534 ± 21 (4)	12.6 ± 0.9 (4)
	DMSO-PSS†	21.5 ± 3.2 (4)	13.7 ± 0.8 (4)	83 ± 0.7 (4)	983 ± 27 (4)	26.4 ± 2.8 (4)
	Control-PSS	6.1 ± 1.1 (3)		347 ± 9.4 (3)	491 ± 16 (3)	9.4 ± 0.3 (3)
	DMSO-PSS‡	14.7 ± 0.9 (3)	14.6 ± 1.0 (3)	96 ± 6.8 (3)	886 ± 7.3 (3)	30.3 ± 3.3 (3)
Unfrozen Experiments	Control-PSS	11.7 ± 1.1 (4)		345 ± 7.0 (4)	536 ± 29 (4)	10.3 ± 0.4 (4)
	DMSO-PSS†	10.6 ± 1.2 (4)	2.0 ± 1.3 (4)	321 ± 3.1 (4)	557 ± 10 (4)	14.8 ± 1.0 (4)

* First step, 10% DMSO-PSS, at 0°C for 5 min, procedure 9(ii).

† First step, 10% DMSO-PSS, at 37°C for 20 min, procedure 9(ii) or 10(ii).

‡ Cooled slowly between -48°C and -70°C, procedure 9(iii).

FIG. 3. The effect of glycerol and DMSO (10%) on uterine wet weight at 24°C.

Horns initially in glycerol (Δ), and control (x——x) in PSS throughout.

Horns initially in DMSO (o) and control (●——●) in PSS throughout.

The change from 10% protective agent to PSS is indicated at the top of the graph.

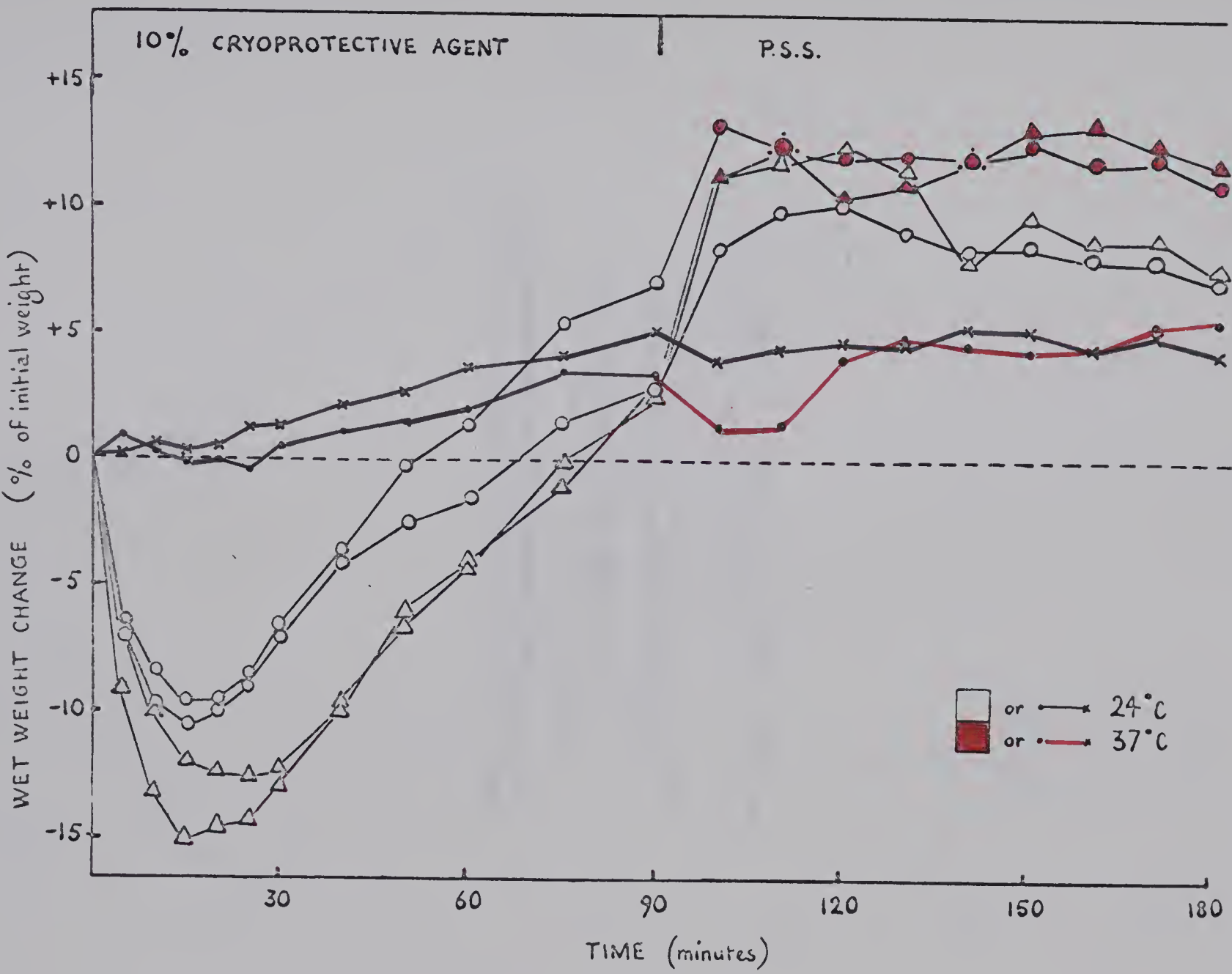

10% CRYOPROTECTIVE AGENT
P.S.S.
+15
+10
+5
0
-5
-10
-15
WET WEIGHT CHANGE (% of initial weight)
30
60
90
120
150
180
TIME (minutes)
or 24°C
or 37°C

FIGURE 3.

FIG. 4. The effect of glycerol and DMSO (10%) on uterine wet weight at 0°C.

Uteri initially in glycerol (Δ) and control (x——x) in PSS throughout.

Uteri initially in DMSO (o) and control (●——●) in PSS throughout.

The change from 10% protective agent to PSS is indicated at the top of the graph.

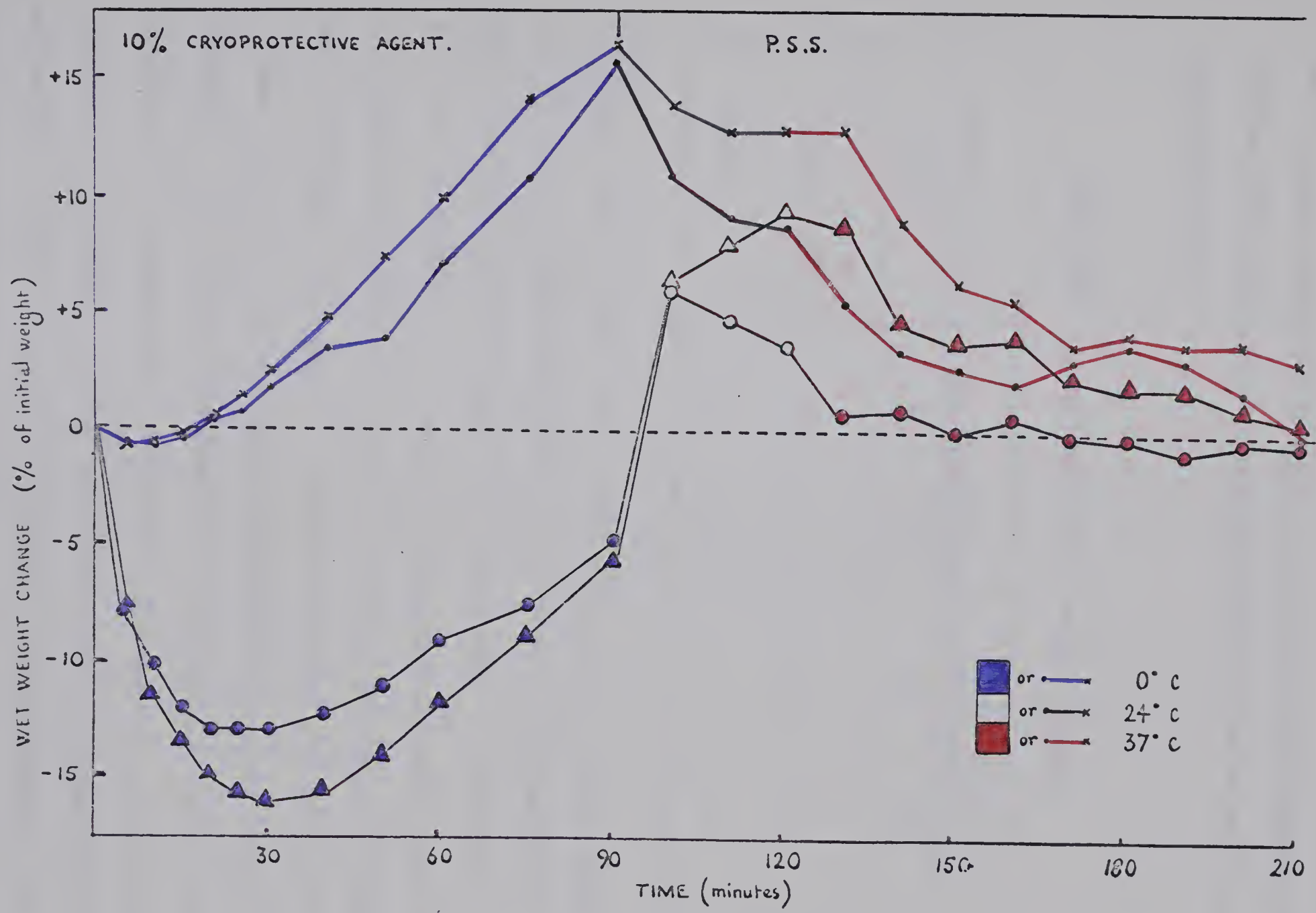

10% CRYOPROTECTIVE AGENT.
P.S.S.
+15
+10
+5
0
-5
-10
-15
(% of initial weight)
WET WEIGHT CHANGE
30
60
90
120
150
180
210
TIME (minutes)
or
0° C
or
24° C
or
37° C

FIGURE 4.

to 0 to 3% on raising the temperature to 37°C. Horns in 10% cryoprotective agent rapidly lost weight, the glycerol ones slightly more so than the DMSO ones, and the loss was slightly greater at 0°C than at room temperature.

TABLE XXII. Comparison of Wet Weight Changes

Cryoprotective Agent	% Wet Weight Loss		Time to Attain Minimum Weight (mins)	
	RT	0°C	RT	0°C
Glycerol	13.5	16	20-25	30
DMSO	10	13	15	25

The time taken to reach these minimum values was a little longer in glycerol than in DMSO and longer at 0°C than at room temperature. After reaching minimum values, the tissues began to increase in weight and it was difficult to see any clear distinction between the effects of glycerol and DMSO on this weight increase; the important difference was between the behavior at room temperature and that at 0°C. At room temperature the wet weight increased until after a total of 90 minutes, the weights approximated to the weights of control horns incubated for a similar time, whereas at 0°C there was a much slower increase of wet weight, so that the tissues never attained the control wet weight, in fact the control curves and the experimental curves were almost parallel during this phase.

The subsequent wet weight changes after removal of the cryoprotective agent were not remarkably different from glycerol and DMSO treated tissues. But it does indeed seem to be a fact that after the cryoprotective agent treatment at 0°C the tissues can return to a more normal wet weight than after the cryoprotective agent treatment at room temperature.

(ii) A Varying Concentration of the Cryoprotective Agent

Experiments, simulating the stepwise changes of concentration of cryoprotective agent which occur during freezing-thawing, took place at 0°C because this was the lowest convenient temperature. Fig. 5 shows that the total loss of wet weight was approximately 27% for both glycerol and DMSO. The stepwise decrease of the cryoprotective agent, either at 5 min intervals at room temperature or at 15 min intervals at 0°C, also revealed no great difference between the effects of glycerol and of DMSO and slight differences were probably related to differences of tissue thickness.

Control horns in PSS at 0°C showed a 10% increase of wet weight after 60 minutes (Fig. 6) and a 26% increase after 180 minutes (Fig. 5) but then subsequently lost some weight during incubation at 37°C. The longer the time at 0°C, the poorer the return of control horns to their original weight.

Fig. 6 shows that, when the concentration of the cryoprotective agent was increased every 5 minutes (instead of 15 minutes) at 0°C, the net loss of wet weight was again approximately 27%. However, when the concentration of the cryoprotective agent was decreased at 5 minute

FIG. 5. Change of uterine wet weight in a varying concentration of cryoprotective agent.

Uteri initially in glycerol (Δ).
Uteri initially in DMSO (o).
Control in PSS throughout (●——●).

The concentration changes of the protective agent are indicated at the top of the graph; arrow A indicates the final change into PSS of experiments using a fast removal of protective agent; arrow B indicates the final change into PSS of experiments using a slow removal of protective agent.

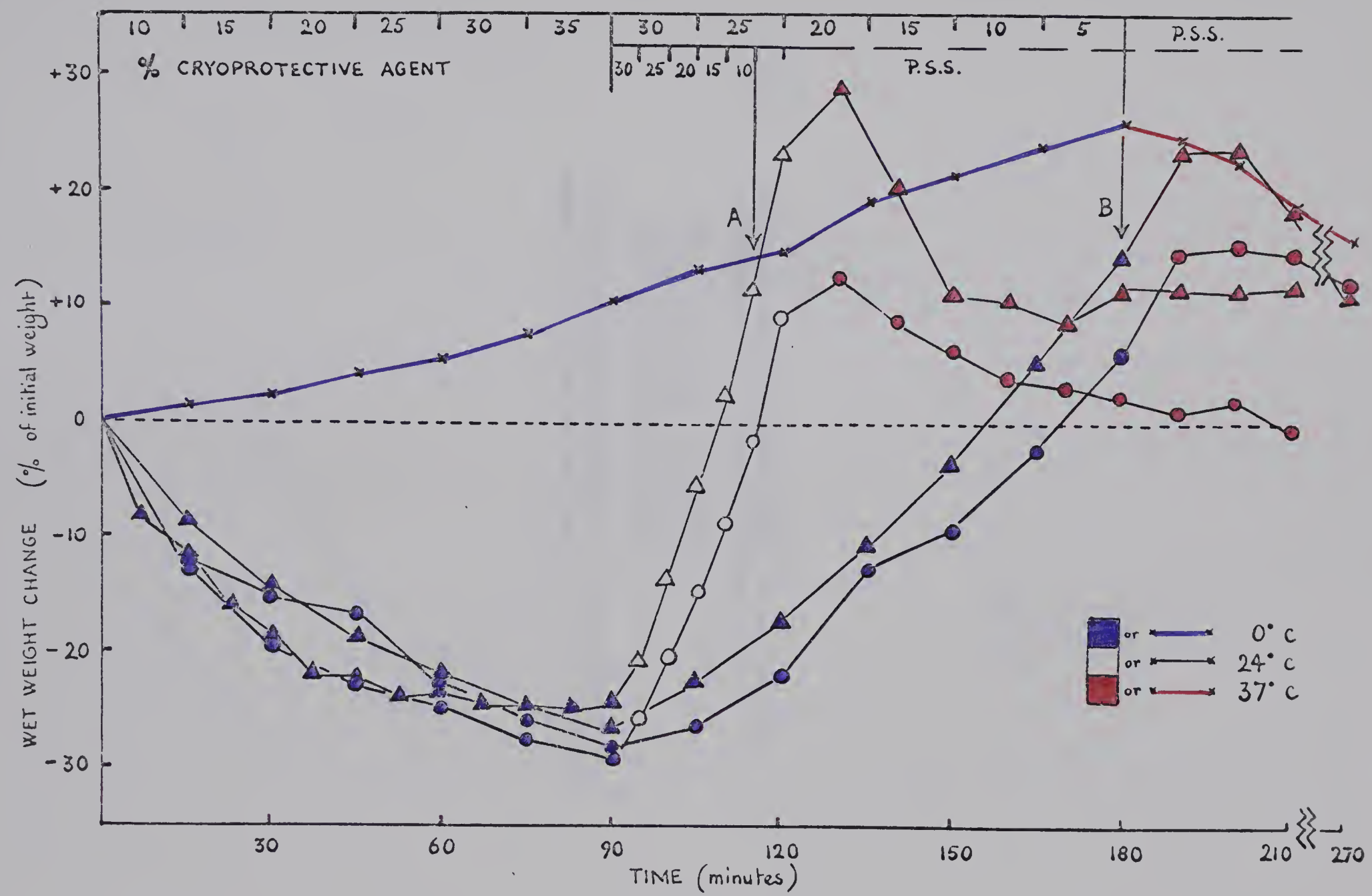

10 15 20 25 30 35
30 25 20 15 10 5
P.S.S.
% CRYOPROTECTIVE AGENT
30 25 20 15 10
P.S.S.
+30
+20
+10
0
-10
-20
-30
WET WEIGHT CHANGE (% of initial weight)
A
B
or 0° C
or 24° C
or 37° C
30
60
90
120
150
180
210
270
TIME (minutes)

FIGURE 5.

FIG. 6. Change of uterine wet weight in a varying concentration of cryoprotective agent.
Uteri initially in glycerol (Δ).
Uteri initially in DMSO (o).
Control in PSS throughout (x——x).

The concentration changes of the protective agent are indicated at the top of the graph.

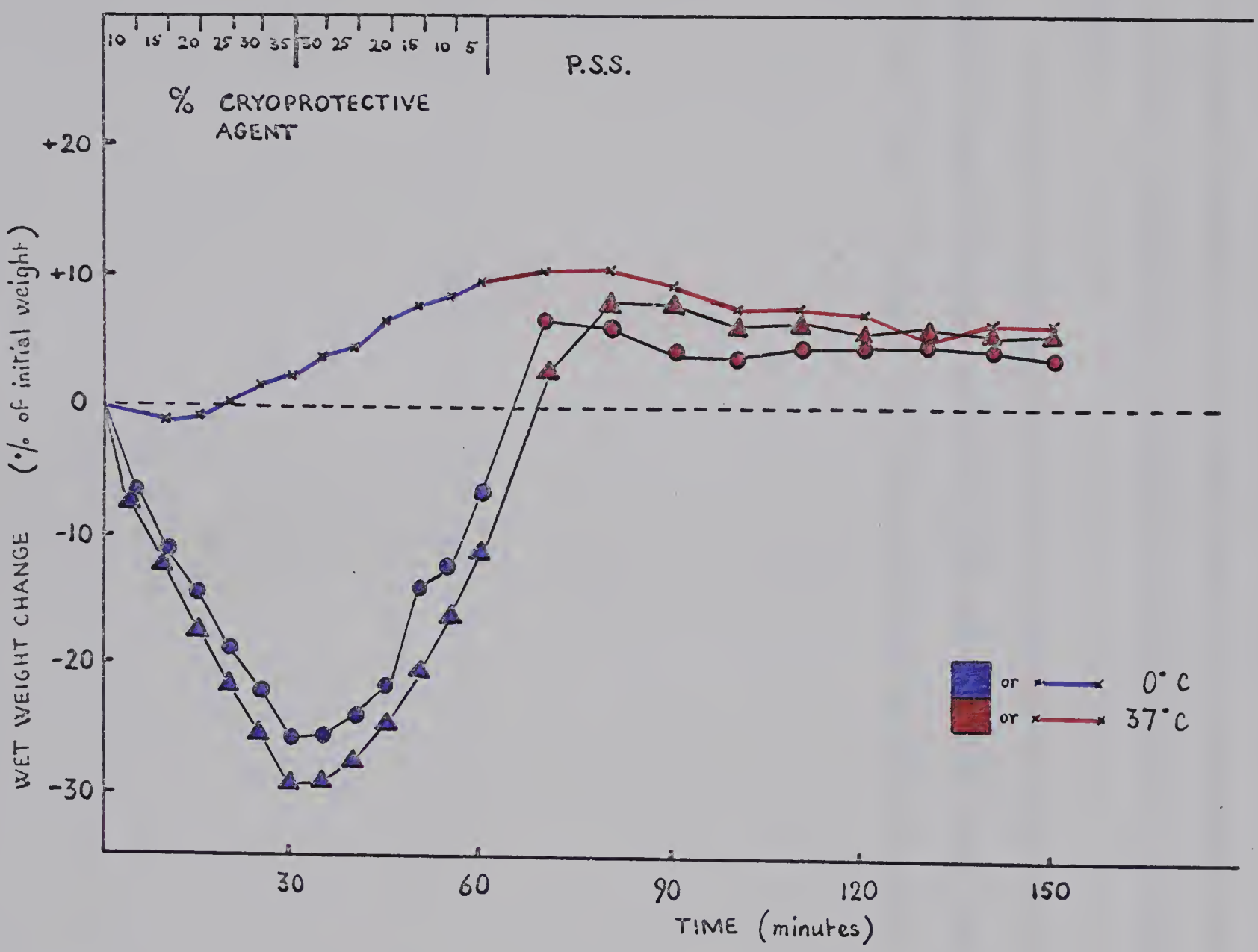

10 15 20 25 30 35 30 25 20 15 10 5
P.S.S.
% CRYOPROTECTIVE AGENT
+20
+10
0
-10
-20
-30
WET WEIGHT CHANGE (% of initial weight)
30
60
90
120
150
TIME (minutes)
or 0° C
or 37° C

FIGURE 6.

intervals at 0°C, the resulting increase of wet weight was less than occurred when this process was carried out at room temperature and less than when it was carried out at 15 minute intervals at 0°C, and is probably related to the shorter time of the increasing concentration phase allowing less intracellular penetration of the cryoprotective agent.

Thus there does not appear to be any great difference, based on wet weight changes, between the use of glycerol and the use of DMSO, but the wet weight changes are affected by temperatures and times.

DISCUSSION AND CONCLUSIONS

1. Damage to Uteri Frozen-Thawed Without Cryoprotective Agent

The results reported in this thesis have shown that uterine horns frozen to -196°C and thawed without any cryoprotective agent underwent severe electrolyte changes (Table IX). That such tissues were unable to contract spontaneously or in response to drugs had previously been shown by Smith (1961) and by Farrant (1964a,b, 1967). After this present work had been completed, Farrant (1967) published electron micrographs of the cellular damage occurring in guinea-pig uteri frozen at -79°C without protective agent, which showed that a large number of the smooth muscle cells lost much of their cytoplasm; the nuclei remained intact but the mitochondria were spherical and greatly swollen.

2. Damage to Uteri Frozen-Thawed with Cryoprotective Agent

Comparison of control horns and of frozen-thawed horns by means of log dose-response curves and by size of spontaneous and drug-induced contractions (Fig. 1, and Table X), indicated that the preservation with glycerol or DMSO using the old incubation methods was completely successful. However, the electrolyte analyses of the control and the frozen-thawed horns (Tables XI and XIII) showed that some potassium was lost from the tissue as a result of the freezing-thawing, and that it was not regained after 1.5 hours, nor even after 3.5 hours of incubation in PSS at 37°C (Table XII), nor was the increased sodium eliminated from the tissues; the net change of tissue sodium and potassium was balanced by the increase of

tissue chloride. Thus one must conclude that electrolyte change is a more sensitive indicator of damage than is the contractility, spontaneous or drug-induced, because normal drug-induced contractions could occur in the presence of a deranged electrolyte content.

Levy, Richards and Persidsky (1962) reported that glycerol caused a 30% loss of rabbit ventricular muscle potassium even in the absence of freezing and thawing. But Levy's experiments were not at all comparable with the procedures used in this thesis, and it can be calculated from Table XIII that in the present experiments the average potassium loss caused by glycerol in the absence of freezing-thawing was 13.5%.

The new methods for glycerol and dimethylsulphoxide using smaller stepwise changes of concentration did not improve the electrolyte balance. The duration of each step was reduced to 15 minutes for the new glycerol method and to only 5 minutes for the new dimethylsulphoxide method, and the particularly bad potassium loss with the new DMSO method indicates that these 5 minute periods were insufficient time for intracellular penetration of the DMSO. Thus intracellular penetration seems to be a factor of great importance. The old and new methods are compared in Table XXIII.

The experiments which simulated those of Farrant, by using a short incubation method (but did not simulate his slow cooling procedure), and in which I exposed the horns to the additional insult of freezing solid at -196°C, instead of retaining in the liquid state at -79°C, subsequently had spontaneous and drug-induced contractions which were very much smaller than those obtained with control horns (Fig. 2B,C). The electrolyte analyses also showed severe changes (Tables XXI and XXIII). The experiments most closely simulating those of Farrant by using identical incubation times

TABLE XXIII. Comparison of Old and New Methods

Protective Method	Concentration Changes % w/v	Time in Each Concentration (mins)	Temp. of First Incubation (°C)	Potassium m mole/kg Dry Wt.	
				Control	Frozen-Thawed
*Glycerol Old	10, 20, 30	45	37	358 ± 44 (10)	165 ± 35 (10)
*DMSO Old	10, 20, 30	45	37	354 ± 16 (4)	168 ± 5.7 (4)
Glycerol New	10, 15, 20, 25, 30, 35	15	0	316 ± 8.0 (6)	111 ± 1.3 (6)
DMSO New	10, 15, 20, 25, 30, 35	5	0	308 ± 7 (2)	73 ± 5 (2)
DMSO New	10, 15, 20, 25, 30, 35	5	37†	383 ± 22 (4)	83 ± 0.7 (4)
DMSO New‡	10, 15, 20, 25, 30, 35	5	37†	341 ± 9.4 (3)	96 ± 6.8 (3)
Glycerol Old + Dipyridamole 5×10^{-6} M	10, 20, 30	45	37	377 ± 14 (8)	257 ± 15 (8)

* Removal of cryoprotective agents by procedure 4(ii).

† First incubation at 37°C for 20 minutes.

‡ Slow cooling procedure (9(iii)); Concentration changes % v/v.

and temperatures, with slow cooling between -48°C and -70°C, but in which the tissue was then frozen solid in liquid nitrogen, also had very reduced tissue potassium (Tables XXI and XXIII). Thus one must conclude that the slow cooling between -48°C and -70°C was not beneficial. Electron micrographs (Farrant, 1967) of tissue treated with short incubations in DMSO, slow cooling to -79°C and no solidification, showed that although the cell membranes were intact, the mitochondria were swollen. Farrant (1967) considers that intracellular penetration of the dimethylsulphoxide may be of no importance, but from the results discussed above, one must conclude that Farrant's supposition is incorrect for tissue which is solidified at -196°C. One cannot say whether Farrant's supposition would apply to tissue only cooled to -70°C.

In Farrant's experiments (1967) with guinea-pig uteri no solidification occurred because the lowest temperature attained (-79°C) was above the eutectic point of DMSO-PSS (-136°C) and these tissues recovered good contractility. However, in his early experiments using 10% DMSO, partial solidification must have occurred and those tissues showed only partial recovery of contractility (Farrant, 1964b, 1965b). Smith (1961) also found that uteri frozen solid with 20% glycerol recovered only poor contractility. It is probable that when solidification occurs, the tissue is subjected to damage additional to that which would occur at the same temperature, if the tissue had not solidified. In my old methods in which the tissues were very rapidly cooled below -8°C, and then frozen solid at -196°C, the electrolyte analyses were the best obtained, and the contractions were normal, in contradistinction to the results obtained by applying Farrant's procedures and then freezing solid at -196°C. Thus Farrant's procedure is not advisable for tissue which is to be frozen solid at -196°C and one must have

grave doubts about the idea that intracellular penetration of the cryoprotective agent is not important.

3. Calcium Changes in Frozen-Thawed Uteri

TABLE XXIV. Collation of Calcium Analyses

Cryoprotective Procedure	Calcium m mole/kg Dry Weight	
	Control	Frozen-Thawed
Nil	9.0 ± 1.2 (2)	26.7 ± 2.0 (2)
Glycerol/New	9.0 ± 0.0 (6)	28.0 ± 0.9 (6)
DMSO/New*	12.6 ± 0.9 (4)	26.4 ± 2.8 (4)
DMSO/New (slow cooling)	9.4 ± 0.3 (3)	30.3 ± 3.3 (3)
Glycerol/Old	10.7 ± 0.5 (4)	21.1 ± 0.4 (4)
DMSO/Old	10.3 ± 0.2 (4)	21.2 ± 1.0 (4)
Glycerol/Old + Dipyridamole (5×10^{-6} M)	9.7 ± 0.5 (4)	17.2 ± 1.4 (4)
	Control	Unfrozen
Glycerol/New	10.1 ± 0.4 (4)	16.7 ± 1.8 (4)
DMSO/New	10.3 ± 0.4 (4)	14.8 ± 1.0 (4)

*Initial step at 37°C.

Table XXIV collates the calcium analyses from different experiments and it is apparent that the new methods result in an increase of tissue calcium as great as occurred in the absence of any cryoprotective agent; the old methods partially prevented the calcium increase but of most interest is the fact that dipyridamole with the old glycerol method of freezing-thawing

resulted in a calcium content only slightly greater than occurred in unfrozen tissue treated with cyroprotective agent.

It is not known if the intracellular calcium, the membrane calcium, or both, were increased. The reason for the increased calcium might be that a "calcium pump" was irreversibly damaged or it might be that during freezing-thawing anionic groups within the membrane were exposed and able to take up calcium ions which subsequently were not lost. An increased tissue calcium resulted from temporary mechanical crushing of the Taenia coli in experiments of Bauer, Goodford and Huter (1965); they found an increase of calcium from 2.6 to 4.9 m mole/kg wet wt. It may be that osmotic changes causing mechanical deformation in the unfrozen tissue is the reason for the somewhat increased calcium content of unfrozen tissue treated with cryoprotective agents.

4. Comparison of Cryoprotective Agents

Although Farrant (1965a) had suggested that the behavior of DMSO and glycerol may be qualitatively different within the cell, and thus it was hoped that DMSO might greatly improve the electrolyte imbalance after freezing-thawing, nevertheless the electrolyte analyses using DMSO showed no improvement over those with glycerol and so cannot give any support to the idea of a qualitative difference of action.

0.5% $MgSO_4$ was not effective as a cryoprotective agent for uteri. Karow (1967) reported that $MgSO_4$ could be used as a cryoprotective agent in the mammalian heart, suggesting that the mechanism of action might be the stabilization of macromolecules during freezing and the prevention of the extraction of bound water, thus preventing denaturation. However,

Karow only cooled the heart to -10°C or perhaps -20°C for 20 minutes. The explanation of Karow's success is not really apparent, but $MgSO_4$ is certainly not a protective agent for uteri frozen at -196°C.

5. Reduction of Tissue Damage as Measured by Electrolyte Balance

The observed electrolyte imbalance after freezing-thawing might arise from damage postulated to occur in one of two ways or by combination of these two ways. Postulate 1: Permanent damage occurred during freezing-thawing such that the cell could no longer function normally. Postulate 2: Temporary changes occurred during freezing-thawing such that essential compounds were lost from the cell, and the cell was subsequently unable to replace them and thus unable to function normally. If the second postulate should apply, then there would be the possibility either of replacing these essential compounds or of trying to prevent their loss. Thus an attempt was made to improve the electrolyte imbalance, whilst not altering the cryoprotective procedure.

(i) Modification of Electrolyte Balance by Adenosine Deaminase Inhibitors

As discussed in the Introduction, an increased ATP content might be associated with an increased potassium content, and might be achieved by the use of the adenosine deaminase inhibitor dipyridamole, and indeed this was found to be so; the potassium loss was reduced from 55% to 32% and the ATP content was increased from 102 to 204 µg/g wet wt. The problem is how did the dipyridamole act? Dipyridamole has been used as a coronary dilator, but although quite a lot has been published about its cardiac action, there is still much confusion, and

a discussion of its coronary action is not applicable to this problem. Possible modes of action of dipyridamole might be to inhibit adenosine deaminase or to affect the permeability of the cell membrane; these are discussed below.

It was shown by Deuticke and Gerlach (1966a) that dipyridamole, RA 171 and RE 102 were competitive inhibitors of cardiac muscle adenosine deaminase at concentrations of 10^{-4} M, but dipyridamole was not a very strong inhibitor and a ratio of dipyridamole:adenosine 10:1 was necessary to obtain about 50% inhibition. Kübler and Bretschneider (1964) (and also Bunag, 1964) found that dipyridamole reduced the permeability of the erythrocyte membrane to adenosine. They also supported their permeability hypothesis by the fact that in a coarse heart homogenate dipyridamole inhibited adenosine breakdown, but did not do so in a fine homogenate. However, Deuticke and Gerlach (1966a) considered these facts may be explained thus; in the coarse homogenate the ratio of dipyridamole:adenosine was such that the adenosine deaminase was inhibited whilst in the fine homogenate the ratio was such that the enzyme was not inhibited.

Gerlach, Deuticke and Duhm (1964) found in human erythrocytes that dipyridamole in a concentration range 10^{-6} to 10^{-4} M inhibited efflux and influx of inorganic phosphate and efflux was the more strongly inhibited. Some of the dipyridamole seemed to be relatively strongly bound to erythrocyte membrane. Kübler (1964) also found dipyridamole inhibited phosphate efflux in erythrocyte. Deuticke et al. (1964) also found dipyridamole (10^{-4} M) inhibited human erythrocyte uptake of glucose, fructose, ribose, mannose, galactose, xylose and arabinose to

varying degrees. Deuticke, Duhm and Gerlach's observations on erythrocytes can be summarized thus:

Dipyridamole Concentration (M)	Compound Inhibited
$> 5 \times 10^{-5}$	Monosaccharides
$> 10^{-6}$	Orthophosphate
$> 10^{-7}$	Adenosine

Whilst it might be possible in the uterine experiments that dipyridamole reduced the phosphate efflux and thus ATP could increase, nevertheless Deuticke and Gerlach (1966a) found with cardiac muscle of dog and guinea-pig that dipyridamole did not reduce the phosphate efflux. Also Kübler (1964) found it did not affect phosphate efflux in dog and guinea-pig auricles.

Deuticke and Gerlach (1966a,b) believed that in guinea-pig heart muscle dipyridamole acts as an inhibitor of adenosine deaminase, based on evidence of both optical density measurements and of chromatographic analyses of adenosine and inosine. This is in contradistinction to Baer, Drummond and Duncan (1966) who said it did not inhibit adenosine deaminase of rat heart at a concentration of 4×10^{-4}M, based only on evidence from an optical density method. However, Deuticke and Gerlach (1966a) drew attention to the fact that Koss (personal communication) found that dipyridamole at concentrations greater than 10^{-4} M interfered

with optical density measurements of adenosine deaminase activity because of its own strong fluorescence. The explanation for the discrepancy between Deuticke et al. and Baer et al. may be that there is a species difference, since Stafford (1966) found a species difference in the action of dipyridamole on the potentiation of the pharmacological effect of adenosine on the heart.

Thus attempting to explain the action of dipyridamole in the uterine experiments, where the effect depended upon the concentration, Tables XIV and XV; it may be that at the lower concentration dipyridamole inhibited phosphate efflux or adenosine loss, or inhibited a deaminase and at the higher concentration, dipyridamole inhibited the uptake of glycerol (analogous to its effects on sugar uptake by the erythrocyte) and thus less protection would be afforded to the cells during freezing-thawing, and thus greater damage might occur.

(ii) Reduction of Tissue Damage by Changing the Calcium Content of the PSS

Smith (1961) and Farrant (1964b) both reported better contractions in frozen-thawed uteri when the calcium content of the physiological salt solution was reduced. Therefore it was of interest to see if tissue damage could be reduced in the present experiments by varying the calcium concentration. Since contractions of the uteri frozen-thawed using glycerol-PSS with 1.4 mM calcium were already as good as those of control tissues (Fig. 1), this parameter of the effect of calcium was not available. When calcium was omitted from the PSS, the tissue potassium was certainly not improved. One might postulate that the beneficial effects found by Smith (1961) and Farrant (1964b) were

due to the lack of calcium affecting the permeability of the cell membranes to the cryoprotective agents. Omitting the PSS calcium certainly increased the tissue wet weight (Table XVI) and also Bozler and Levine (1958) found a small amount of calcium reversed the swelling of smooth muscle in isotonic sucrose or in distilled water. However, although one might consider that the permeability to the cryoprotective agent was affected in a similar way to the water permeability, Farrant (1965a) has shown that in the Taenia coli, the kinetics of DMSO permeation were not affected by omission of the calcium from the PSS. Thus the explanation for Smith's and Farrant's observations is still obscure. Possibly more damage occurred in their tissues than to my tissue, damage allowing calcium to interfere with the functioning of the contractile proteins.

The reason for the greatly reduced tissue potassium after freezing-thawing in the presence of high calcium is hard to explain and since high calcium itself in the absence of freezing does not cause this potassium loss (Table XVI) it may be that during the freezing-thawing, structural alterations are "cemented" by the calcium present in high concentration and that these structural changes in some way affect the efficiency of the potassium pump. Such an explanation may also be the reason why magnesium sulphate used with glycerol also caused a great loss of tissue potassium; both are divalent cations. Unfortunately instrumentation for calcium estimations was not available at the time of these experiments and thus it is not known if there is any correlation between the tissue calcium and the PSS calcium.

(iii) Effect of Dibucaine

Dibucaine (a local anesthetic) has been shown to reduce the hemolytic action of hypotonic solutions on erythrocytes and hence it may stabilize the erythrocyte membrane (Seeman, 1966). Spherules of phospholipid plus cholesterol are to some extent analogous to biological cell membranes and Bangham (1967) found these spherules had certain permeabilities to cations and that the cation diffusion rate could be reduced by local anesthetics.

Thus there was some reason to see if dibucaine might stabilize the cell membrane and improve the tissue potassium of frozen-thawed uteri. However, at a concentration of 10^{-6} or 10^{-4} M, dibucaine did not reduce the potassium loss from frozen-thawed tissue, and at 10^{-6} M it was not harmful (Table XVIII).

6. Weight Changes of Uteri

The irreversible wet weight changes of the frozen-thawed tissues might result from impairment of the metabolic processes due to damage during freezing-thawing, but since damage might also arise from the severe osmotic changes incurred with the use of cryoprotective agents, the wet weight changes in unfrozen tissue were therefore investigated. However, in addition to the osmotic effect, the wet weight changes observed in the unfrozen tissues (Figs. 3 and 4) might also result from temporary inhibition of the metabolic processes due to cooling or due to the cryoprotective agent.

Osmosis initially causes a loss of wet weight when the tissue is immersed in hypertonic cryoprotective-PSS and then as the agent gradually penetrates intracellularly, so this osmotic effect decreases and water

reenters the cells and they regain weight. Then when the tissue is suddenly transferred into PSS, it swells due to the osmotic effect of the cryoprotective agent within the cells. Thus the wet weight increase in Fig. 3 is a crude indicator of the intracellular penetration of the cryoprotective agent. Because the wet weight changes in 10% glycerol and 10% DMSO were very similar at 24°C, it seemed that glycerol and DMSO penetrated uterine smooth muscle at similar rates at 24°C. This is not in agreement with the general opinion that DMSO penetrates much more rapidly than glycerol as discussed in the Introduction and is certainly not in agreement with Farrant's findings in the guinea-pig Taenia coli (Farrant, 1965a).

It is impossible to say whether or not the cryoprotective agent was able to penetrate intracellularly at 0°C, since experimental curves were parallel to control curves during the weight increase phase at 0°C (Fig. 4). There was also little difference between the use of glycerol and of DMSO when stepwise addition and decrease of the cryoprotective agent was used at 0°C (Figs. 5 and 6). The increasing weight during stepwise removal of the cryoprotective agent was due to the agent's lessening osmotic activity and one must not conclude that this increasing wet weight was due to the cryoprotective agent penetrating intracellularly. Although from Fig. 4 there is uncertainty about the ability of the cryoprotective agents to penetrate intracellularly at 0°C, yet comparison of Figs. 5 and 6 shows that the regained weight was greater in Fig. 5, in which the tissues had been subjected to increasing cryoprotective agent concentration for 1.5 hours, than in Fig. 6, in which increasing cryoprotective agent concentration was for 0.5 hour, which thus suggests that longer time had allowed greater intracellular penetration of the cryoprotective agent. However, it is also possible that

longer time in cryoprotective agent caused greater damage and thus a greater wet weight increase. If indeed the cryoprotective agent could not penetrate at 0°C, this may be the explanation for the greater damage (as measured by potassium loss) occurring in the new DMSO method (Tables XXI and XXIII) than in the old DMSO method (Tables XIX and XXIII).

Removal of the cryoprotective agents at 37°C is probably undesirable because at that temperature the noxious effects due to the agents would be greater than at a lower temperature. The subsequent incubation in PSS theoretically would be best at 37°C instead of 24°C, because faster metabolism would allow a quicker reversal of the swelling; however, Fig. 3 does not support the idea that 37°C is better.

The crudeness of the wet weight method does not allow evaluation of glycerol and DMSO permeation kinetics, but does allow some practical comparison of these agents.

Studies of intracellular penetration of cryoprotective agents by calculation of intracellular concentrations from extracellular volumes is fraught with difficulties due to three factors:

(i) The cryoprotective agent causes changes of the intracellular and extracellular volumes during the time taken to reach a steady state.

(ii) Non-penetrating agents such as inulin used to determine the extracellular volume, take a long time to attain equilibrium, during which time the cryoprotective agent may damage the tissue.

(iii) Solutes used to determine the extracellular volume, and which are

capable of some intracellular penetration, will be affected by a phenomenon observed both in living tissue and in artificial membranes, in which the osmotic activity of some other substance (e.g., the cryoprotective agents) can increase the influx of the solute (Franz, William and Van Bruggen, 1968). These difficulties may account for certain questionable results obtained by Farrant (1965a). Whilst kinetic studies of influx/efflux of radioactive cryoprotective agent might prove helpful, the most promising and simple method of investigation seems to be to perfuse or superfuse with radioactive cryoprotective agent and measure the time taken for the effluent radioactivity to return to the normal level.

SUMMARY

It was found that the old method of protecting uteri with glycerol or dimethylsulphoxide was superior to any of the new methods using shorter incubation times and smaller concentration changes. Thus one concluded that intracellular penetration of the cryoprotective agent was of great importance for tissue frozen solid at -196°C. This is contrary to the opinion of Farrant and the divergence of opinion may be due to the fact that Farrant did not solidify his tissue; he only cooled to -79°C.

A tenuous deduction concerning the possible beneficial effect of dipyridamole in the freezing-thawing process was confirmed by potassium and ATP analyses. Possible explanations of the beneficial action can be deduced but no evidence is yet available.

Finally, investigations of tissue wet weight changes in cryoprotective agents did not indicate any appreciable difference between the actions of glycerol and dimethylsulphoxide.

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